Sulfur Allocation and Vanadium−Sulfate Interactions in Whole Blood Cells from the Tunicate *Ascidia ceratodes*, Investigated Using X-ray Absorption Spectroscopy

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Sulfur K-edge X-ray absorption spectroscopy (XAS) has been used to investigate the distribution of sulfur types in two whole blood cell samples, in selected subcellular blood fractions, and in cell-free plasma from the tunicate *Ascidia ceratodes*. Whole blood cells are rich in sulfate, aliphatic sulfonate, and low-valent sulfur. The sulfur K-edge XAS spectrum of washed blood cell membranes revealed traces of sulfate and low-valent sulfur, but no sulfate ester or sulfonate. Sulfonate is thus exclusively cytosolic. Cell-free blood plasma contains primarily sulfate sulfur. Gaussian fitting and sulfate quantitation for two whole blood cell samples, each representing dozens of individuals from separate collections taken per annum, yielded average sulfur type concentrations for the two populations: (first year, second year): [sulfate], 110 mM, 150 mM; [sulfonate], 99 mM, 70 mM, and; [low-valent sulfur], 41 mM, 220 mM. On titration of sulfate with acid or V(III) in aqueous solution, the strong 2482.4 eV 1s → (valence t_2) sulfur K-edge XAS transition of tetrahedral SO_4^{2-} splits into 1s → a_1 and 1s → e transitions, because HSO_4^- and VSO_4^+ are of C_{3v} symmetry. Gaussian fits and appropriate comparisons allow the following assignments: (compound/complex, 1s → a_1 (eV), 1s → e (eV)): *myo*-inositol hexasulfate, 2480.8, 2482.8; HSO_4^-, 2481.4, 2482.7; VSO_4^+, 2481.2, 2482.9. The energy separating the a_1 and e states of complexed sulfate appears to be solvation dependent. From these studies is derived an explicit inorganic spectrochemical model for biological V(III) and sulfate. The average endogenous equilomer concentrations of sulfate complexed with V(III) and/or H^+ within the two blood cell samples are calculated from this model. The results provide a natural explanation for the observed biological broadening of *A. ceratodes* blood cell sulfur K-edge XAS spectra.

Introduction

In previous work we and others[1−14] have reported on the presence and chemistry of sulfur in whole blood preparations of the ascidians, including *Ascidia ceratodes*. These organisms exhibit a remarkable blood chemistry, which in recent years has been the subject of considerable study (for recent reviews, see refs 15−18).

The Phlebobranch ascidians sequester vanadium(III) within up to three types of blood cells.[11,16,19−21] In *A. ceratodes* the average observed blood cell vanadium concentration of ca. 0.1−0.2 M[6,11,22,23] represents a [V] increase by a factor of about 1−2 million above ambient seawater. A blood cell [sulfur] of 0.25 M was also found in this organism[6] for a biological enhancement of about 9-fold. Electron beam X-ray microprobe studies of single fixed blood cells have also identified intracellular sulfur and vanadium in *A. ceratodes*[11] and in other ascidians both within granules[1,4,5,9,24] and within vacuoles[2,3,21] for various cell types.

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Using sulfur K-edge X-ray absorption spectroscopy (XAS), we have directly observed endogenous sulfur within living whole blood cell packs from *A. ceratodes*, with further resolution according to oxidation state and chemical type.[7,8,14] Evidence for intracellular disulfides, for an aliphatic sulfonic acid, and for sulfate ion was found.

Blood cell sulfate has been quantitated by gravimetry as $BaSO_4$,[2,6] in addition to direct detection by sulfur K-edge XAS. However, the sulfonate in blood cells from *A. ceratodes* was observed only as a numerically generated difference XAS spectrum,[8] or it was inferred from second-derivative sulfur K-edge XAS spectra of whole blood cell packs, or it was deduced from Gaussian curve fitting of such XAS spectra.[14] A cysteate-like sulfonate feature has been identified in the Raman spectrum of blood cell homogenates from *Ascidia gemmata*.[13] Though direct spectroscopic or chemical identification of blood cell sulfonic acid is lacking, sulfur K-edge XAS spectroscopy can readily audit any endogenous sulfonic acid.

We have thus examined a sulfate-free blood cell cytosolic lysate, a blood cell membrane preparation, and cell-free blood plasma from the combined blood from 120 *A. ceratodes* specimens. The question of the aggregate hematological allocation of sulfur types between aqueous cytosolic and membranous environments is thus specifically addressed.

To access questions of sulfur biochemistry at the population level, we have examined whole blood cell samples which included the combined blood from dozens of individuals taken from each of two autonomous cohorts, which were sequentially collected per annum from the same locale (Monterey Bay, CA). Gaussian curve fitting of sulfur K-edge XAS spectra of whole blood cells was combined with gravimetric quantitation of blood cell sulfate to calculate, compare, and report for the first time the average cytological concentrations of sulfate, sulfonate, and low-valent sulfur within the two independent *A. ceratodes* blood cell samples.

The sulfur K-edge XAS spectrum of inorganic sulfate is shown to titrate with added acid or vanadium(III) in new high-resolution experiments. Gaussian deconvolution and appropriate comparisons are used to assign the principal bound-state transition features in the spectra of *myo*-inositol hexasulfate, and the HSO_4^- and VSO_4^+ complex ions. From these data an explicit inorganic spectrochemical model for blood cell vanadium(III), sulfate, and acid is constructed. This model is used to explain the observed biological broadening of the sulfur K-edge XAS spectrum of whole blood cells in terms of endogenous bisulfate and VSO_4^+ complex ion. On the basis of this model, the average blood cell concentrations of these species are calculated and compared for the above two cross-year blood cell samples. Information regarding the *in vivo* concentrations, types, allocation, fates, and biochemistry of blood cell sulfur is presented.

Materials and Methods

Specimens of *A. ceratodes* were collected from the Monterey Bay Yacht Harbor, Monterey, CA. They were maintained in aerated and periodically refreshed seawater at 4 °C until use (2−6 days). Animals selected for experiment were considered healthy by the responsiveness of the incurrent and excurrent siphons to stimulus, by the presence of a firm integument, and by the observation of a strong heartbeat. Two autonomous collections of *A. ceratodes* were made. The first, on June 1, 1985, was used to produce sample "S85", consisting of the combined blood cells from 53 animals. The second, on May 27, 1986, was used to produce sample "S86" and included the combined blood from about 85 animals. The May 27, 1986 collection also provided the blood cell sample used to prepare the subcellular blood fractions (see below), which represented whole blood cells from 120 individuals. Blood was removed by cardiac puncture using sterile disposable 1 cm^3 syringes as described previously[6,8] and kept on ice prior to processing.

Washed whole blood cells, made free of surface sulfate, were prepared as described previously.[14] Since it was our initial intention to obtain K-edge XAS spectra of biological chloride as well as biological sulfur, it was necessary to remove both chloride and sulfate ions adsorbed to the external surface of the blood cells. Thus, whole blood cell packs were washed with an isotonic ($\mu = 0.710$ M) metal formate synthetic blood plasma, buffered to pH 6.6 with 17 mM imidazolium trifluoroacetate.[8,14]

The subcellular fractions of whole blood cells were prepared from the blood of 120 animals from the S86 collection. Whole blood was combined and centrifuged (5000*g*, 10 min), to yield 1.00 ± 0.03 cm^3 of packed cells. The relatively high centrifugation force was chosen to maximize the hematocrit. Inspection of the sample revealed no color change or other evidence of lysis (*i.e.*, blue or brown specks) following this step. The cell-free plasma supernate was removed, and the cells were lysed by the addition of 5 mL of anaerobic pH 2 trifluoroacetic acid (TFA), followed by rapid stirring with, and reflux within, a wide-mouth Pasteur pipet. The deep reddish-brown lysate was centrifuged as before, and the clear brown supernatant liquid was removed. This was followed by three similar centrifugation washes of 2 mL each, using pH 2 anaerobic trifluoroacetic acid. Each wash was combined with the initial supernatant solution. The total volume of the acid solution of the soluble blood fraction was made to be 12.00 mL.

Sulfate-free lysate was prepared from the 12.00 mL soluble fraction above, by the addition of 0.50 mL of 1 M barium trifluoroacetate solution. The clear brown solution became opaque, and the barium sulfate precipitate was removed by centrifugation (5000*g*, 10 min). A further 100 μL of the barium salt solution added to the clear brown supernate caused no further precipitation. The sulfate-free lysate was neutralized from pH 1.6 to 1.9, at which point a green flocculent precipitate began to form. Neutralization was discontinued, and the lysate was shell-frozen and lyophilized. The brittle, glassy solid thus resulting was crushed to a green powder, which was stored at −20 °C in sterile plastic tubes.

The barium sulfate precipitate obtained above was washed several times by resuspension in deionized water followed by centrifugation (3000*g*, 10 min). The faintly colored solid was then lyophilized and weighed, amounting to 34.4 ± 0.1 mg of $BaSO_4$. Analysis for vanadium and total volatiles (as water) was carried out by Desert Analytics, Tucson, AZ 85717. For the solid $BaSO_4$ sample, loss on drying was 1.0%, and [V] was ≤0.08%.

The membrane fraction, following the three trifluoroacetic acid washes described above, consisted of deep green material, pelleting to 5.5 mL on centrifugation (5000*g*, 20 min). This was washed once further with 4.5 mL of isotonic ($\mu = 0.710$ M) sodium phosphate, pH 6.6, to neutralize the pellet and stabilize the membranes. The suspension was centrifuged at 5000*g* for 20 min, following which the pellet volume decreased from 5.5 mL to about 2 mL. The supernatant was colorless.

Cell-free blood plasma was obtained by centrifuging a whole blood sample at 3000*g* for 10 min. The clear colorless supernate was removed into a sterile plastic tube using a Pasteur pipet.

Lyophilized blood lysate controls were solutions of 20 mM methanesulfonic acid and 100 mM (*i.e.*, 5× excess) sodium, barium, or vanadium(III) trifluoroacetate, respectively, in pH 2 solution. The solution included 1.0 M glucose as a dispersive matrix, and 1 mg/mL of bovine serum albumin (BSA).[25] Each solution was shell-frozen and lyophilized.

Barium trifluoroacetate solution was prepared by treating a suspension of 9.86 g of barium carbonate in triply deionized water with 10.14 g of anhydrous trifluoroacetic acid and diluting the resulting clear colorless solution to 50.0 mL with triply deionized water.

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Vanadium tris(trifluoroacetate) 1.0 M stock solution was prepared by catalytic reduction of 2.27 g (12.5 mmol) of vanadium pentoxide suspended in 8.60 g (75.4 mmol, 5.80 mL) of trifluoroacetic acid in 25 mL of total aqueous solution. The reduction was carried out anaerobically using two 100 mg charges of 10% Pd/C and using a dihydrogen gas stream, as described earlier.[6] Electron paramagnetic resonance (EPR) analysis of the vanadium tris(trifluoroacetate) solution by the method of standard additions of $VOSO_4$ indicated the presence of 1.7 mM residual vanadyl ion. EPR spectra were taken using a Varian E-112 Century series instrument, with samples kept at *ca*. 140 K using a quartz liquid-nitrogen flow-dewar.

Vanadium tris(methanesulfonate) 1.0 M stock solution was prepared by means of anaerobic catalytic reduction, in a manner similar to that described above, using 2.27 g (12.5 mmol) of vanadium pentoxide suspended in 25.0 mL of 3.030 M methanesulfonic acid (75.8 mmol) prepared from 98% methanesulfonic acid (Aldrich Chemical Co.). EPR analysis of the 1.0 M $V(O_3SCH_3)_3$ stock solution as outlined above indicated a residual vanadyl ion concentration of 1.5 mM.

Solutions for X-ray spectroscopic titration were prepared at 67 or 100 mM $[S]_{total}$ to obviate the possibility of self-absorption, evident at $[S] \geq 200$ mM. Inorganic sulfate solutions were prepared by appropriate pH adjustment of a 0.10 M solution of $NaHSO_4$. Sulfate solutions containing vanadium(III) were prepared anaerobically in a nitrogen-filled Lexan glovebox (Plas-Labs, Inc., Lansing MI) by serial admixture of 67 μL of 1.0 M $NaHSO_4$ with appropriate amounts of the 1.0 M $V(CF_3CO_2)_3$ stock solution (see above) and 3.0 M potassium trifluoroacetate, pH 1.8, to make a total volume of 1.00 mL. Each solution was adjusted to pH 1.79 $\pm$ 0.05 within the Lexan glovebox using a Beckman model 3500 pH meter.

Solutions of methanesulfonic acid with vanadium(III) were likewise prepared anaerobically by appropriate admixture of the above solutions of 1.0 M vanadium tris(trifluoroacetate) and 3.0 M potassium trifluoroacetate, with 67 μL of 1.0 M sodium methanesulfonate solution in 15 mM methanesulfonic acid. The final solutions were adjusted to pH 1.79 $\pm$ 0.03 as above. Solutions without added vanadium(III) were prepared by appropriate pH adjustment of a 0.10 M solution of methanesulfonic acid.

Sulfur K-edge XAS spectra of whole blood cells and the subcellular blood fractions were measured at the Stanford Synchrotron Radiation Laboratory (SSRL) under dedicated operating conditions at 3.0 GeV and 30−60 mA ring current, using the 8-pole wiggler beamline 4-2 with a wiggler field of 18 kG, in unfocused mode. The Si[111] double-crystal monochromator was detuned 66% at 2740 eV to minimize harmonic contamination. Data were measured between 2420 and 2740 eV with a step size of approximately 0.15 eV in the edge region (2450−2490 eV). Spectrometer resolution was approximately 0.5−0.6 eV, and spectra were calibrated using the 2472.02 eV peak of thiosulfate.[26] At least two calibration scans were taken during each fill.

Sulfur K-edge XAS spectra of the inorganic sulfate and methanesulfonate solutions were likewise measured at SSRL on 54-pole wiggler beamline 6-2 under dedicated operating conditions of 3.0 GeV and 40−90 mA with a wiggler field of 5.0 kG. The X-ray beam was energy-resolved using a Si[111] double-crystal monochromator, detuned 25% or 32% at 2740 eV.

Background subtraction was performed by fitting a polynomial to the pre-edge region and subtracting this polynomial from the entire spectrum. Data were then normalized by fitting a polynomial spline to the region above the edge and normalizing to an edge-jump of 1.0 at 2490 eV. The washed blood cell spectrum is the average of 11 scans. The spectrum of the lyophilized lysate is the average of 4 scans, and that of the membrane fraction is the average of 3 scans. The sulfur K-edge XAS spectrum of cell-free plasma was obtained using the 54-pole wiggler beamline 6-2, operating in undulator mode, with a device field of 1.45 kG. The Si[111] double-crystal monochromator was detuned 26% at 2740 eV. The data are the average of 2 scans.

Blood product samples were maintained on ice until measurement, and the spectra were obtained at ambient temperature. The blood cell samples were visually inspected for lysis, as evidenced by blue or brown specks, or discolored patches, both before and after data acquisition. No such evidence was seen.

Gaussian fits to sulfur K-edge spectra were carried out using the program FITCUR as described in detail elsewhere.[14] Control fits using solutions of known composition indicated an analytical fit error of about $\pm$10%,[14] which was applied to the sulfur type quantitation arising from the fits to the blood cell sulfur K-edge spectra reported herein.

Results and Discussion

The experimental results described here extend and complete an analysis begun earlier,[14] in which *A. ceratodes* blood cell samples collected across an 18 month span were examined for biological sulfur. In this report sulfur concentrations are quantitated in terms of specific sulfur types within blood cell samples obtained from collections of *A. ceratodes* designated S85 and S86,[14] which were obtained from the same locale 1 year apart (see Materials and Methods for details). Sample S85 represented blood from 53 animals, and sample S86 represented blood from about 85 animals. The whole blood sulfur K-edge XAS spectra of these samples query the blood chemistry at the population level. That is, the results point to differences in aggregate blood chemistry between populations of animals separated by time, rather than pointing to distribution of sulfur species among the individual blood cell types.

In addition, sulfur types are pursued through various subcellular blood fractions obtained from a whole blood cell sample of the S86 collection representing 120 individuals. These data again reflect the aggregate storage locale of blood cell sulfur, rather than the eccentricities of variation between individuals.

Preparation of the *A. ceratodes* subcellular blood fractions discussed herein is outlined in the scheme depicted in Figure 1. The fractions track the distribution of sulfur among blood cell cytosol (as cleared lysate), blood cell membranes (as pelleted lysate), and cell-free blood plasma. The cleared lysate fraction represents the cytosol freed of endogenous sulfate to facilitate the K-edge XAS observation of any blood cell sulfonic acid. The cytosolic and membrane fractions represent the aggregate biological partitioning of sulfur between hydrophilic and lipophilic environments, respectively. Sulfur K-edge XAS examination of these fractions further divides the resident sulfur into various types defined by structure and oxidation state.

Whole Blood Cells. Sulfur K-edge X-ray absorption spectroscopy involves the set of 1s $\rightarrow$ valence transitions of the sulfur absorber. The selection rules which normally govern electronic transitions apply to XAS spectra. Therefore, the shape and energy position of the XAS spectrum can yield specific information about the chemical environment and oxidation state, respectively, of sulfur.[7,26−28]

The absorption maxima of the K-edge XAS spectra of sulfur of valence 0 through 6+ monotonically shift to higher energy at 1.6 $\pm$ 0.2 eV per unit formal oxidation state change, over a total range of 13 eV.[7,8] Similar relationships have also been found between sulfur oxidation state and transition energies in sulfur X-ray emission spectra.[29,30]

The sulfur K-edge XAS spectrum of a sample of whole blood cells from collection S86, representing 85 animals, is shown in Figure 2a, and the second derivative of the XAS spectrum is shown in Figure 2b. The cells were washed free of surface sulfate. The common abscissa in Figure 2 is marked to show

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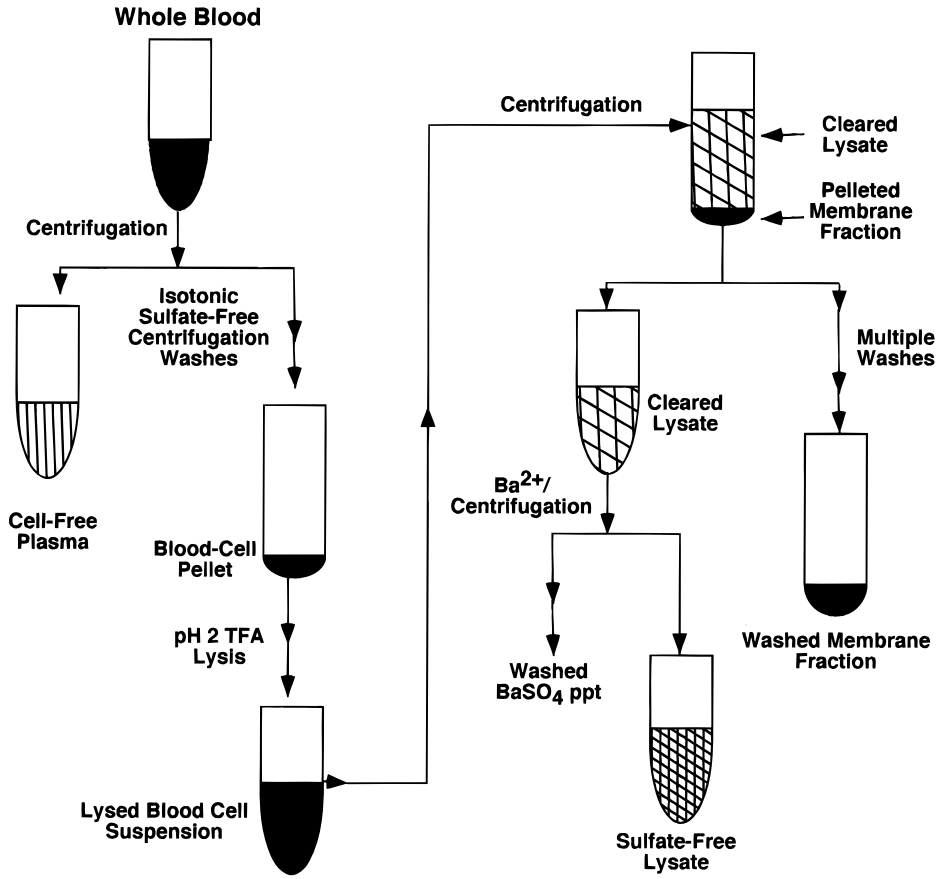

Figure 1. Scheme outlining the experimental sample preparation. The samples were constructed to reveal the relative amounts and types of sulfur in the following blood products: washed whole blood cells, washed blood-cell membranes, blood cell cytosolic lysate made free of sulfate, and blood-cell-free plasma. The sulfate-free lysate sample tests the predicted presence of blood cell aliphatic sulfonate.

the energy regions corresponding to the K-edge absorption maxima of sulfur valences 0 through 6+.

The second-derivative minima at 2481.1 and 2482.4 eV imply sulfur oxidation states of 5+ and 6+, representing a sulfonic acid and sulfate ion, respectively. However, these signals are broadened relative to the XAS spectrum of these ions in neutral aqueous solution.[8,14] The minima near 2473 and 2474 eV may represent the presence of one or more endogenous disulfides. These data have been discussed in more detail elsewhere.[14] Unusual polysulfide metabolites have been isolated from the colonial ascidian *Polycitor* sp.,[31] which may be relevant to the disulfide signal. Further experiments are planned to test and elucidate this finding.

Blood Cell Sulfonate. In earlier work, the blood cell sulfonic acid signature was extracted from XAS difference spectra, or was deduced by comparisons with model solution XAS spectra, or by Gaussian curve fitting experiments to whole-cell sulfur K-edge XAS spectra. Since this finding was novel, and because any whole blood cell sulfur K-edge spectrum of sulfonate sulfur is badly obscured by that of sulfate, it was deemed worthwhile to independently verify the presence of this sulfur type. In addition, sulfate esters exhibit a K-edge spectrum which includes strong well-separated features at 2480.8 and 2482.8 eV (see below). These features can be mistakenly interpreted to represent a mixture of sulfonic acid and acid sulfate, which have their principal absorption features near these respective energies. Direct visualization of the sulfur K-edge XAS of any endogenous sulfonic acid is therefore necessary to forestall this error.

To directly view the spectrum of biological sulfonate, sulfate ion was quantitatively removed as the barium sulfate precipitate from the supernate of the centrifuged lysate from a whole blood cell pack obtained from the S86 collection. Analysis of the solid $BaSO_4$ obtained revealed only traces ($\leq$80 ppm) of vanadium. The sulfate-free supernatant was then lyophilized.

The sulfur K-edge XAS spectrum of this material in Figure 3 is unambiguously that of an aliphatic sulfonic acid.[8,32] There is no evidence of the sulfur K-edge spectrum from any residual sulfate ester. However, compared with the XAS spectrum of methanesulfonic acid, that of the lysate-derived sulfonate is strongly broadened with lower intensity features which are shifted slightly in energy. The broadening is not likely to be due to self-absorption (see below). The XAS signature of a small amount (*ca.* 15%) of low-valent sulfur appears at 2473 eV.

The Figure 3 (inset) second-derivative XAS spectrum confirms the complete loss of sulfate (compare Figure 2b) and exhibits features near 2479 and 2481 eV which are not present in the XAS spectrum of free methanesulfonate. As discussed below, these features are most likely due to complexation of sulfonate by vanadium(III) in the lyophilized lysate. Vanadium analysis of whole blood cells taken from the S86 collection of animals indicated an average blood cell [V] = 79 mM.[14]

To directly evaluate the possibility of a V(III)−sulfonate interaction in the lyophilized lysate, powder models were prepared by lyophilization of solutions of methanesulfonate with glucose/BSA in the presence or absence of vanadium(III). Figure

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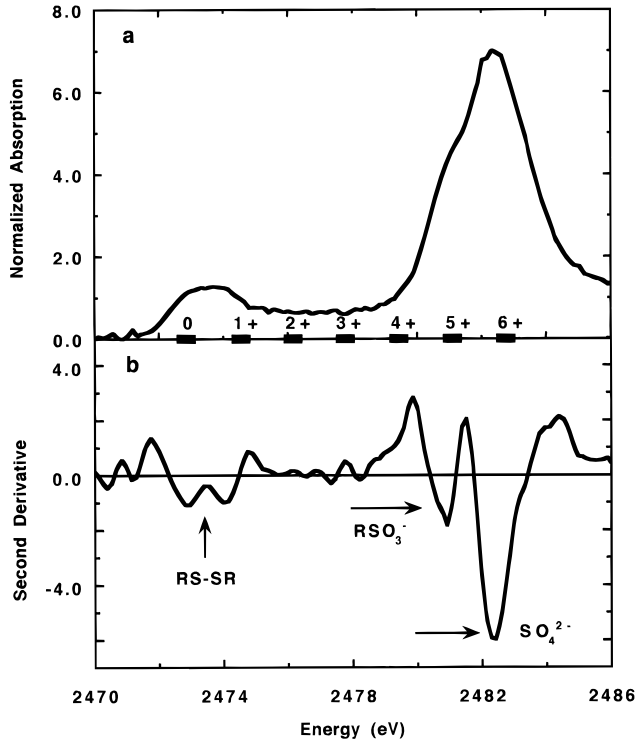

Figure 2. (a) Sulfur K-edge XAS spectrum of a whole blood cell preparation from 85 specimens of *A. ceratodes* from collection S86 (see text). (b) Second derivative of the blood cell spectrum, where features due to the various sulfur types in the absorption spectrum are more easily observed. The second-derivative features have been labeled as to the assigned sulfur type. The central abscissa is marked to indicate the XAS spectral energy position of the K-edge maximum of sulfur of various oxidation states.[8]

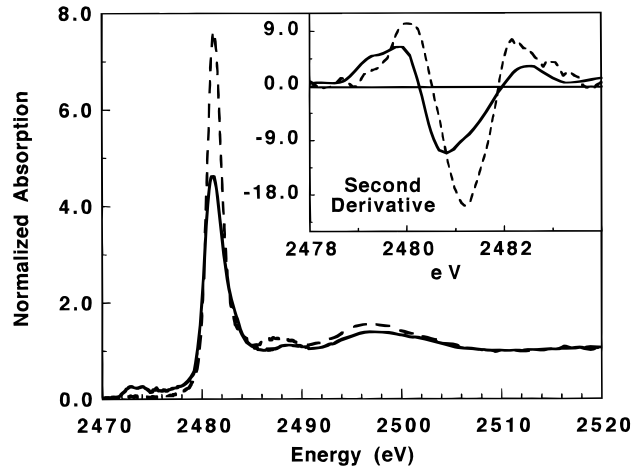

Figure 3. Sulfur K-edge spectra of (—) lyophilized sulfate-free cytosol and (– – –) a 100 mM pH 3.0 solution of methanesulfonic acid. The lysate was prepared from a second whole blood cell sample taken from the S86 collection. Soluble sulfate was removed as $BaSO_4$. The major absorption features of the lysate spectrum show an energy shift and broadening relative to the same features in the sulfonic acid XAS spectrum. Inset: The second-derivative XAS spectrum of (—) the sulfate-free lysate sample and (– – –) the methanesulfonic acid solution. Differences in position and intensity of the main features are here more evident. Note the complete loss of the sulfate signal at 2482.4 eV (*cf.* Figure 2), indicating the quantitative removal of this ion. There is no indication of any resident sulfate ester.

4a,b shows that complexation with vanadium(III) has clearly broadened the sulfur K-edge XAS absorption and second-derivative spectra of methanesulfonate, indicated by the vertical

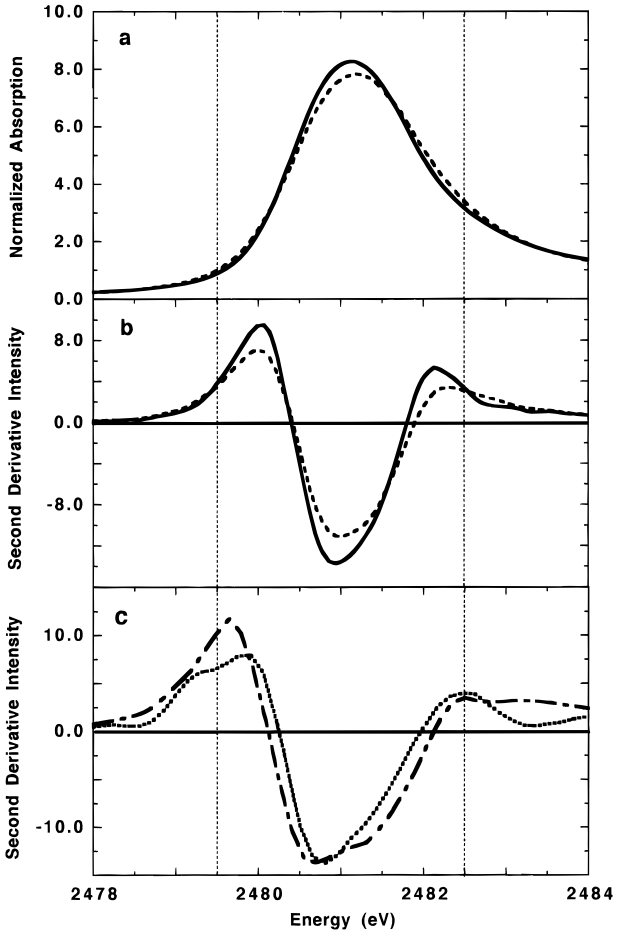

Figure 4. Sulfur K-edge XAS spectrum of (a) (—) aqueous sodium methanesulfonate and (– – –) a pH 2.0 solution of vanadium(III) and methanesulfonic acid, each lyophilized in a glucose/BSA matrix. (b) The second derivatives of the XAS spectra in part a. The line types have the same significance. Substitution of barium for sodium had no effect on the sulfur K-edge spectrum. (c) The second-derivative XAS K-edge spectra of (···) 1.0 M V(III) tris(methanesulfonate) in 15 mM methanesulfonic acid and (—·—) lyophilized blood cell lysate. Broadening due to the formation of the $[V(O_3SCH_3)_n]^{(3-n)+}$ complex ion is obvious in the region of 2479.5−2482.5 eV (vertical dashed lines). Other features due to V(III)−sulfonate interactions are evident.

dashed lines near 2479.5 and 2482.5 eV. A control mixture substituting Ba^{2+} for V(III) yielded methanesulfonate K-edge XAS and second-derivative spectra which were superimposable with those of the sodium salt. Thus the effect of added vanadium(III) is seen to be unique and most likely arises due to the presence of a specific V(III)−OSO_2CH_3 bond.

The broadening of the sulfonate XAS spectrum from lyophilized lysate is clearly greater than that of the lyophilized model. This implies that a larger proportion of sulfonate is complexed by vanadium(III) in the lysate. Thus Figure 4c compares the second-derivative sulfur K-edge XAS spectra of the lyophilized lysate and 1.0 M $V(O_3SCH_3)_3$ in acidic solution. The high total concentration in the 1.0 M solution ensures an observable amount of liganded sulfonate as the complex ion $[(H_2O)_{6-n}V(O_3SCH_3)_n]^{(3-n)+}$, and the XAS spectrum is strongly broadened as a result.

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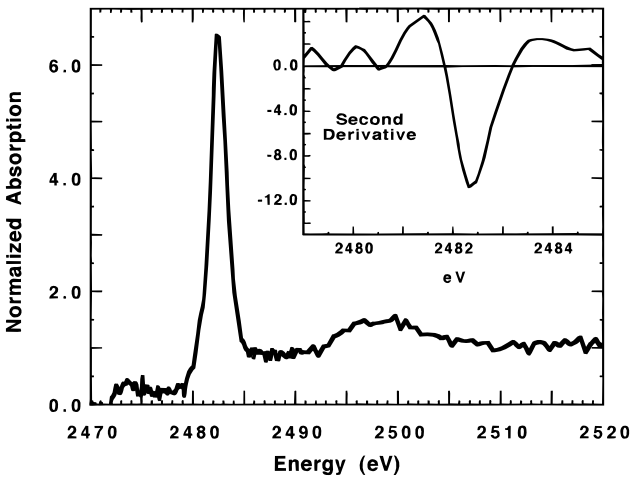

Figure 5. Sulfur K-edge XAS spectrum of the centrifugable (membrane) subcellular fraction. The high noise level is indicative of a low (<5 mM) sulfate concentration, which persisted despite extensive washing. The signal from a small concentration of membrane-bound low-valent sulfur is evident at 2473 eV. Inset: The second derivative of the high-valent portion of the spectrum. No evidence for membrane-bound sulfonolipids or sulfate esters is observable at 2481 eV.

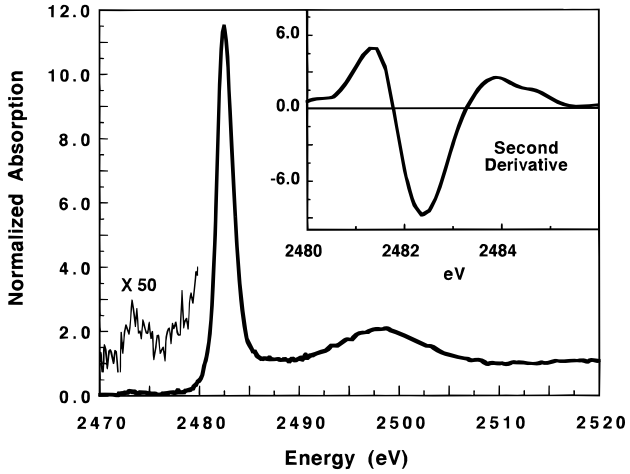

Figure 6. Sulfur K-edge XAS edge spectrum of the cell-free blood plasma from *A. ceratodes*. The spectrum is essentially that of pure sulfate. A trace of low-valent sulfur is evident near 2473 eV, and this signal is also shown expanded for clarity. Inset: The second derivative of the high-valent sulfur edge region of the spectrum. The complete absence of any observable sulfonic acid feature at 2481 eV is noteworthy (compare Figure 2).

The spectroscopic features of the lysate sample can also imply complexation of sulfonate with other liberated biogenic Lewis acids such as Fe(III) or VO^{2+}. However, these possibilities can be excluded since no other transition metals were found present in sufficient quantity in *A. ceratodes* blood cells to account for the magnitude of the observed result.[6,33,34] In addition, acidic and anaerobic conditions of lysis prevent autoxidation of liberated V(III)[23,34−36] and, by analogy, any Fe(II) (see below).

The sulfur K-edge X-ray spectrum of the membrane fraction of lysed whole blood cells is given in Figure 5. This spectrum reflects neutral inorganic sulfate, present despite several washing steps. The signal-to-noise of the spectrum is consistent with an average concentration of sulfate in the low (≤5) mM range.

This sulfate could be nonspecifically associated with the membranes despite the several washes, or it may indicate some sort of specific sulfate-binding locus, or protein.[37,38] The membrane fraction also produced a small but observable signal from low-valent sulfur, accounting for about 19% of the total sulfur in the sample.

The data of Figure 5 excludes membranal sulfated lipopolysaccharides as the source of cellular sulfate. These compounds are known to be abundant in ascidian tunic.[39−43] Other ascidian-derived sulfate esters[44,45] are also now excluded from blood cell membranes. Sulfate esters are readily distinguished from sulfate by sulfur K-edge XAS, as discussed below (*cf*. Figure 8). Likewise, there is no evidence of membrane-associated lipophilic sulfonic acids, as have been found in other marine organisms.[46,47]

The cell-free blood plasma contained considerable sulfate, as indicated by the strong signal-to-noise ratio of the sulfur K-edge XAS spectrum given in Figure 6. Gravimetric analysis as barium sulfate yielded a plasma sulfate concentration of 22.4 mM. This value is comparable to previous findings (see below) and to [sulfate] in the plasma of other ascidians.[48] The second derivative of the high-valent sulfur portion of this spectrum (inset) is cleanly indicative of sulfate only. However, the very weak feature at 2473.5 eV does indicate a small concentration of soluble low-valent sulfur, perhaps reflecting plasma proteins.[25,49−53]

Since neither the membrane fraction nor the cell-free plasma gave any evidence of sulfonate ion, then the observed blood cell sulfonate either was transported rapidly into the appropriate blood cell(s) following synthesis elsewhere or else was produced within blood cells. Taurine has apparently been found in ascidians,[17,54] and soluble sulfonates have been found in a variety of marine algae[46] and animals.[55,56] Cysteate-like sulfur has been reported (but not isolated) in ascidian blood cell homogenates using Raman spectroscopy.[13]

Complexation of Sulfate and Sulfonate. Association of vanadium(III) and sulfate has been inferred to occur at low pH

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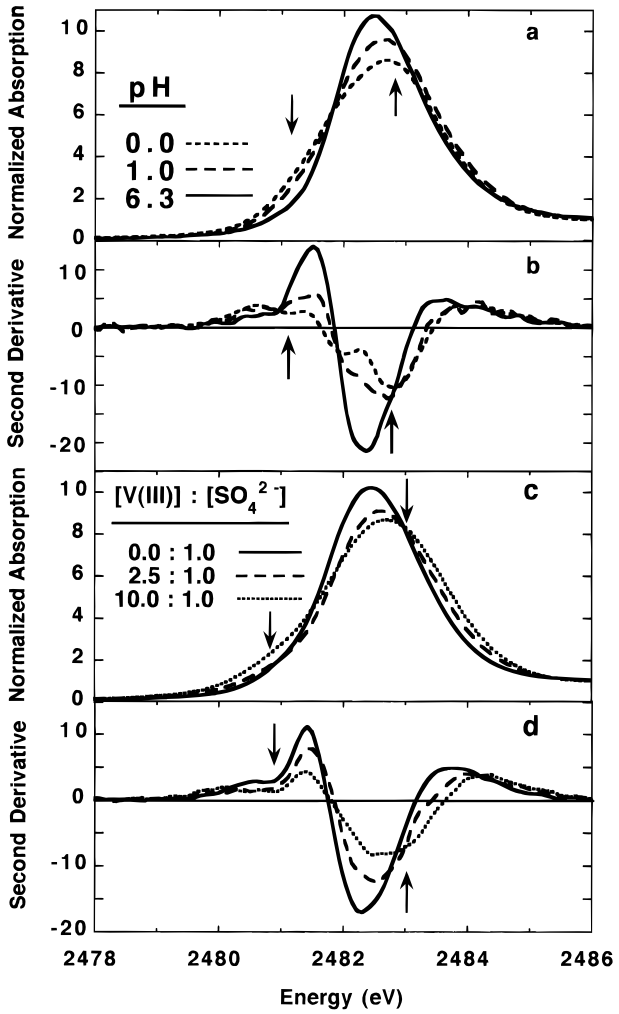

Figure 7. Modification of the K-edge XAS spectrum of solution sulfate: (a) as a function of pH; (b) second derivative of the spectra in part a. The pH of each solution is given on the face of part a. At pH 0.0, virtually all of the sulfate is present as the bisulfate ion, HSO_4^-. (c) Modification as a function of [V(III)]. (d) Second derivatives of the spectra in part c. The ratios of vanadium and sulfate are given on the face of part c. About 99% of the sulfate is calculated to be within the VSO_4^+ complex ion in the 10:1 vanadium(III):sulfate solution.[57] Arrows mark the regions where the spectroscopic consequences of increasing titrant are most obvious. The strong solution bisulfate feature at 2482.0 eV in part b and the small minimum near 2482.5 eV for VSO_4^+ in part d, are due to the emergence of a minor absorption feature at each respective energy.

within intact blood cells from *A. ceratodes*,[8,14,23,25] and we have suggested that this association accounts for the biological broadening of the sulfur K-edge XAS spectra of blood cells from this animal. To systematically investigate this process, the sulfur K-edge XAS spectra of methanesulfonate and sulfate ions in inorganic solution were titrated with vanadium(III) (at pH 1.8) or with acid. The experimental solutions were 67 or 100 mM in total sulfur, respectively, avoiding the problem of self-absorption which limited the resolution of earlier work.[14]

Titration of sulfate with acid under these conditions (Figure 7a,b) produced new sulfur K-edge XAS absorption and second-derivative features near 2481.1 and 2482.8 eV. These were well developed at pH 0.0, where about 99% of the sulfate is present as equilibrium bisulfate ion. The strong shoulder at 2482.0 eV in the second-derivative spectrum of pH 0.0 sulfate is fortuitous and corresponds to a surprisingly small feature at that energy in the XAS absorption spectrum.

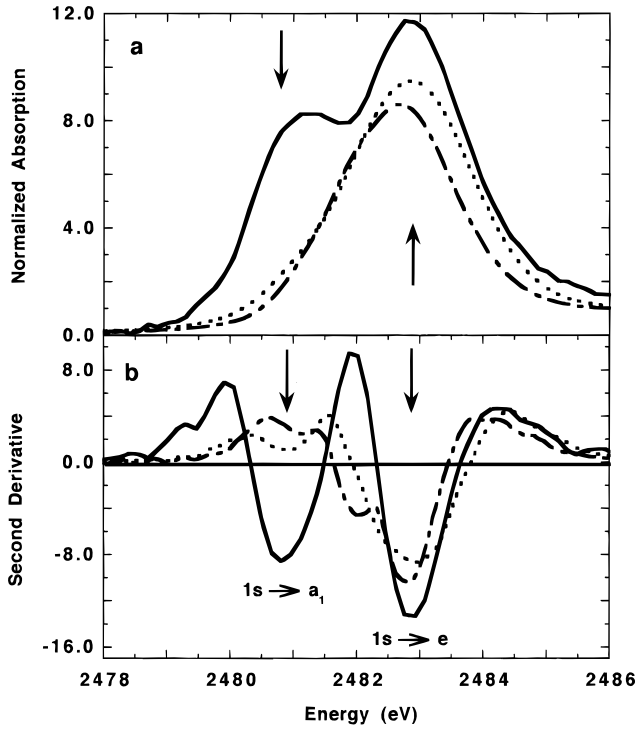

Figure 8. Sulfur K-edge XAS spectra of (a) (—) *myo*-inositol hexasulfate, 25 mM solution; (···) aqueous $[(H_2O)_5VSO_4]^+$; and (—·—) aqueous HSO_4^-. (b) Second derivatives of the XAS absorption spectra in part a. The sulfate ester groups in *myo*-inositol hexasulfate are of strictly C_{3v} symmetry. Thus the $1s \rightarrow a_1$ and $1s \rightarrow e$ transitions are exclusively present and are marked with arrows at 2480.8 and 2482.8 eV, respectively. The near coincidence in energy of the second-derivative XAS features in the spectrum of *myo*-inositol sulfate with those in the spectra of VSO_4^+ and HSO_4^- permit the assignment of the $1s \rightarrow a_1$ and $1s \rightarrow e$ transitions in the XAS spectra of these latter species.

On titration of sulfate with vanadium(III) in pH 1.8 solution, effects analogous to those produced by acid were observed at 2480.8 eV and at 2483.0 eV (Figure 7c,d). The small shoulder at 2482.5 eV in Figure 7d (1:10 SO_4^{2-}:V(III)) is analogous to the above-mentioned fortuitous bisulfate feature at 2482.0 eV in Figure 7b. From the equilibrium constants governing V(III) and sulfate[57] and the K_a of HSO_4^- (1.20×10^{-2} M), about 99% of the sulfate in the 1:10 SO_4^{2-}:V(III) solution is within the $[(H_2O)_5VSO_4]^+$ complex ion.

These XAS spectral changes can be understood in terms of the spectroscopic consequences of the symmetry-lowering of sulfate ion on complexation. In the K-edge XAS of free sulfate ion there is only one $1s \rightarrow$ (valence t_2) transition, observed at 2482.4 eV, under T_d (tetrahedral) symmetry.[26,27,58,59] On protonation, or on complexation with vanadium(III), the symmetry of sulfate ion is lowered to C_{3v}. The triple degeneracy of the t_2 final state is thereby split into an a_1 state and a doubly degenerate e state.[26,27,59] The $1s \rightarrow a_1$ and $1s \rightarrow e$ transitions in bound sulfate produce two new absorption features at slightly different energies, observed as broadening.[14,26,59−61] Similar effects have

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been noted in the Raman spectra of sulfate on complexation: with V(III) in solid $KV(SO_4)_2$[60] and with V(III) and VO^{2+} in aqueous solution.[62,63]

Figure 8 compares the absorption and second-derivative sulfur K-edge XAS solution spectra of HSO_4^- and VSO_4^+ ions with those of *myo*-inositol hexasulfate. Since *myo*-inositol hexasulfate includes six covalent sulfate esters, it is a good example of covalently bound purely C_{3v} symmetry sulfate. The 1s → a_1 and 1s → e transitions in the sulfur K-edge XAS spectrum of *myo*-inositol hexasulfate should exhibit an intensity ratio which reflects the relative 1:2 orbital degeneracy ratio, and they are thus assigned to the features at 2480.8 and 2482.8 eV (arrows), respectively.

Comparison of the absorption and second-derivative sulfur K-edge XAS spectra of both bisulfate and the VSO_4^+ complex ion with that of *myo*-inositol hexasulfate (Figure 8a,b) allows assignment of the 1s → a_1 and 1s → e transitions in the spectra of these equilibrium-complexed sulfate ions by analogy.

Gaussian fitting experiments were used to deconvolute the C_{3v} transition features of sulfate observed in the sulfur K-edge XAS spectrum of *myo*-inositol hexasulfate, and following protonation, or complexation with vanadium(III). Four Gaussian elements were found necessary to reproduce these spectra, and the results of the fits are given in Table 1.

The magnitude of the splitting between the a_1 and e orbital states is likely to be a measure of the distortion of sulfate ion away from pure tetrahedral symmetry. From Table 1, this distortion is indicated to be in the order sulfate ester > VSO_4^+ > HSO_4^-. This point merits further discussion.

The crystal structures of ionic metal sulfates reveal that the S−O bond length varies only slightly in various crystalline environments, averaging 1.482 ± 0.003 Å.[67] Extensive hydrogen bonding does not greatly affect this length, *e.g.*, 1.46−1.47 Å in dihydrazinium sulfate.[68] The O−S−O angles, another measure of sulfate distortion, vary from the ideal tetrahedral value of 109.5° by about ±2° in crystallographic ionic sulfate.

In the crystal structure of organosulfate esters the unique $^-O_3S-OR$ bond length is 1.61 Å, for an increase of about 0.13 Å. The remaining bond lengths concurrently decrease: by about 0.01 Å in potassium ethyl sulfate,[69] or by about 0.04 Å in tyrosine *O*-sulfate.[70] Likewise in ammonium bisulfate the unique $^-O_3S-OH$ bond length is 1.55 Å,[71] and it is 1.60 Å in sodium bisulfate.[72]

Table 1. Results of Gaussian Fitting to Sulfate Forms

sulfate form	center of Gaussian (eV)	fwhm[a] of Gaussian (eV)	rel area	1s → valence transition[b]	ΔeV (e − a_1)
SO_4^{2-} ion	2482.1	0.80	0.20	ua[c]	
in pH 6.3 soln	2482.5	1.51	5.0	t_2	
	2483.7	1.38	1.0	Ryd[d]	
	2484.0	arctan		Ryd[d]	
$[(H_2O)_5V^{III}O-SO_3]^+$	2481.2	1.99	1.0	a_1	
in pH 1.8 soln	2482.2	1.12	0.21	ua[c]	1.7
	2482.9	2.02	4.1	e	
	2483.9	arctan		Ryd[d]	
$HO-SO_3^-$ ion	2481.4	1.39	1.0	a_1	
in pH 0.0 soln	2481.9	0.71	0.12	ua[c]	1.3
	2482.7	1.85	6.1	e	
	2483.5	arctan		Ryd[d]	
$R_3CO-SO_3^-$ as	2480.8	1.45	1.0	a_1	
myo-inositol	2481.4	1.00	0.21	ua[c]	2.0
hexasulfate in	2482.8	1.90	2.3	e	
25 mM soln	2483.4	arctan		Ryd[d]	

[a] Full width at half-maximum. [b] 1s → valence transitions in the sulfur K-edge XAS spectra of VSO_4^+ and HSO_4^- were assigned by analogy with the spectrum of *myo*-inositol hexasulfate. [c] Unassigned. [d] Fit component used to model the Rydberg envelope (see text).

In some contrast, for sulfate liganded to V(III),[66,73] to VO^{2+},[74,75] or to Fe(III),[76] the length of the $^-O_3S-OM$ bond is only 1.48−1.51 Å (where M = transition metal ion).

These crystallographic data imply that the magnitude of the a_1 and e orbital energy splitting in the sulfur K-edge XAS spectra of singly bound sulfate species should be in the order sulfate ester ≥ bisulfate ≫ (O_3S-OV(III)). This result is contrary to the order observed (Table 1) and may reflect the XAS experimental conditions, *i.e.*, the liquid solution state.

In acidic aqueous solution, sulfate has complete rotational freedom. The orientational relaxation times (*i.e.*, the end-over-end rotation) of nitrate and carbonate, for example, are in the range of a few picoseconds in water solution.[77,78] From X-ray scattering experiments, sulfate is known to be hydrogen bonded to about eight water molecules[79−81] in both neutral and acidic aqueous solution. The solvent cage of water molecules and the attendant hydrogen-bonding network surrounding sulfate ion is likewise dynamic on the picosecond time scale.[82] Thus a more approximately tetrahedral symmetry may be restored to bisulfate in acidic aqueous solution by virtue of a position-averaged hydrogen-bonding and solvent cage. In acidic aqueous solution these dynamics may lift much of the unique length found in the solid state $^-O_3S-OH$ bond by averaging the $^-O_3SO-H$ interactions over all four sulfate oxygens. For example, the Raman spectrum of pH 0.2 sulfate showed no sign of the split peak expected if the T_d symmetry of sulfate were reduced to C_{3v} on protonation in solution.[62] The fact that such splitting is observed at all in the sulfur K-edge XAS spectrum of solution bisulfate may be due to the short lifetime of an electronic transition relative to a Raman transition.

Position averaging of the sulfate oxygens may not be possible within a transition metal sulfate complex ion. The results of X-ray scattering experiments on solutions of the $FeSO_4^+$ complex ion, which is homologous to VSO_4^+, are best fitted by a model which includes a nontumbling sulfate ligand.[79] If solvent or rotational averaging processes are less likely to be

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available to sulfate within the solution VSO_4^+ complex ion, then the $^-O_3S-OV(III)$ bond might retain its unique character on a time-scale governed by the dissociation kinetics of VSO_4^+. This effect would account for the ordering observed in the relative energy splitting of the $1s \rightarrow a_1$ and $1s \rightarrow e$ transitions, as noted above. The average lifetime of the $FeSO_4^+$ complex ion in solution is about 40 ms.[83,84]

These equilibrium processes may also explain the observation that the $1s \rightarrow a_1$ and $1s \rightarrow e$ transition features in the sulfur K-edge XAS solution spectra of both pH 0.0 sulfate and vanadium(III) sulfate do not exhibit the 1:2 intensity ratio expected from the relative orbital degeneracies.

Similar but greatly attenuated effects were noted in the sulfur K-edge XAS spectra of aqueous methanesulfonate solutions on titration with acid or with vanadium(III). Since the basicity of the alkylsulfonate ion is very much weaker than that of sulfate ($pK_a \sim 0$),[85] the spectroscopic consequences pertaining to complexation by proton or vanadium(III) were diminished.

Sulfur Quantitation within Blood Cells. In a previous study,[14] we elucidated the *in vivo* ratios of biological sulfate and sulfonate in three independent *A. ceratodes* blood cell samples using Gaussian fits to whole blood cell sulfur K-edge XAS spectra. The widths of the Gaussians reflected whether the biological sulfate or sulfonate species were free ions in solution, were protonated, or were bound to vanadium(III) within a complex ion.

Here we extend this earlier analysis and comparison by complete quantitation of blood cell sulfur, with specification of sulfur type for the whole blood cell samples S85 and S86, which were collected from the same locale 1 year apart (see Materials and Methods for details).

In Figure 9 we show the Gaussian fit to the high-valent region of the sulfur K-edge XAS spectrum of a whole blood cell sample from collection S86. This spectrum is the same as that of Figure 2 and was fitted with a total of 6 parameters: the primary $1s \rightarrow$ valence transition of blood cell sulfate and sulfonate represented by one Gaussian each; a minor Gaussian near 2481 eV; two Gaussians representing the Rydberg envelope arising from higher energy bound state transitions within these ions; and a linear offset to account for the total edge-jump of the low-valent sulfur.

The Gaussians used to fit the S86 XAS spectrum were consistent with blood cell sulfate and sulfonate in pH 1.5 solution only,[14] indicating the absence of an *in vivo* biological vanadium(III) complex ion. Therefore, any vanadium(III) present in this whole blood cell sample must have been sequestered away from contact with either biological sulfate or sulfonate. Absent in the whole cells, the association of sulfonate with V(III) in the lyophilized lysate as described above (*cf.* Figures 3 and 4) was thus after blood cell lysis.

In a sulfur K-edge XAS spectrum, the relative height of the ionization edge-jump is proportional to the concentration of each sulfur type in the sample. The areas of the sulfate and sulfonate Gaussians used in the fits were also found to be proportional to concentration.[14] This permitted the ratio of blood cell sulfur types to be directly determined from the areas of the Gaussians used to fit the sulfur K-edge XAS spectrum of each whole blood cell sample.

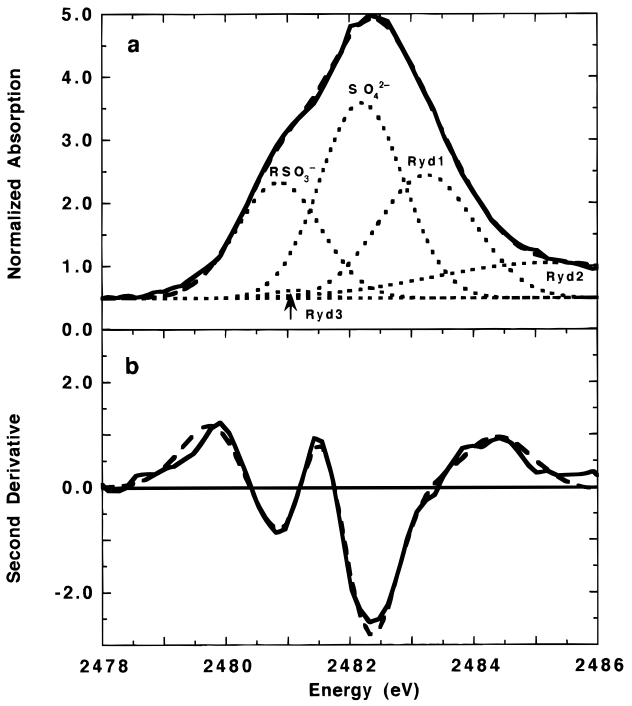

Figure 9. Sulfur K-edge XAS spectra of (a) (—) the high-valent portion of the blood cell spectrum of Figure 2; (— — —), the fit to the spectrum; and (---) the Gaussian components of the fit to the spectrum. The arrow points to a minor Gaussian at 2481 eV which remains unassigned. See text for a discussion of the other assignments. (b) The second derivatives of (—) the blood cell sulfur K-edge XAS spectrum and (— — —) the fit to the spectrum.

For the S85 blood cell sample, Gaussian analysis yielded blood cell ratios for sulfate:sulfonate:low-valent sulfur of 1.0: 0.90:0.37 (sulfate:sulfonate ratios ± 10%, see Materials and Methods). Soluble sulfate in this sample was quantitated as $BaSO_4$,[6] yielding an average in vivo concentration of 110 ± 3 mM.

For the S86 blood cell sample, the Gaussian curve fit indicated a ratio of 1.0:0.5:1.5 (±10%) for endogenous sulfate:sulfonate: low-valent sulfur. Following the addition of Ba^{2+} to the lysate obtained from 1.00 mL of packed cells (from 120 animals), 34.4 mg of barium sulfate was obtained. The recovered $BaSO_4$ is equivalent to an average intracellular concentration of about 150 mM sulfate. Analysis of the barium sulfate precipitate indicated an upper limit of 80 ppm for any vanadate contaminant.

The soluble sulfate fraction accounts for virtually all of the blood-cell sulfate. Therefore the analytical results for sulfate combined with the ratio of sulfur types from the Gaussian fits allow calculation of the average concentrations of all of the sulfur types in each of the two blood cell samples. These concentrations represent a biological variation in sulfur across a full year for the resident *A. ceratodes* population. The results for each sample were produced using the same techniques and, thus, include the same set of assumptions and conditions. The results of this analysis are given in Table 2.

Given the large number of animals represented by each blood cell sample, it seems unlikely that the variations in sulfur distribution and concentrations given in Table 2 reflect scalar differences between individual animals. Instead, the results point to aggregate variations at the population level, presumably reflecting a community response to environmental cues. Thus for the S85 blood cell collection the soluble [sulfate] was 110 ± 3 mM, and total average [sulfur] was found to be 250 ± 2 mM.[6] Within the blood cells of the S86 *A. ceratodes* collection,

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Table 2. Comparison of Blood Cell Sulfur Concentrations Across 1 Year

sample[a]	S85[b]	S86
[sulfate] (mM)	110	150
[sulfonate] (mM)[c]	99	70
[low-valent sulfur] (mM)[c]	41	220
total $[sulfur]_{cell}$ (mM)[c]	250	440
$[sulfate]_{plasma}$ (mM)[d]	20.2	22.4

[a] The detailed Gaussian fit for blood cell sample S85 has been reported.[14] [b] The blood cell sulfur concentrations for the S85 collection are slightly greater than previously reported[6] because they have been recalculated to reflect [total sulfur] rather than [soluble sulfur] for the purposes of this comparison. The XAS method queries total cellular sulfur, whereas the lysate contains only soluble sulfur. Some of the sulfate and low-valent sulfur remain membrane-bound after lysis and washing (vide infra). [c] The analytical precision of [sulfate] is about ±3%, and it is about ±10% for the remainder (see Materials and Methods). [d] The plasma sample was rendered cell-free by centrifugation at 3000*g*. Seawater [sulfate] ~27.6 mM.[90]

soluble [sulfate] was 150 ± 5 mM and total average [sulfur] was 440 ± 44 mM. Hence a striking 1.8-fold increase in average organismal level of blood cell sulfur is recorded in the course of a single year.

From Table 2, the 1 year increase in soluble [sulfate] by 40 mM is moderate and is comparable in absolute magnitude to the decrease in sulfonate concentration by about 29 mM. The major *in vivo* sulfur concentration change resides in the nearly 6.5-fold increase in low-valent [sulfur]. This latter finding indicates that a large amount of thiol/disulfide sulfur was directed into blood cells during the intervening year. Alternatively, the change in sulfur ratios may reflect a dramatic change in the distribution of blood cell types.[86] In either case, these results reinforce the suggestion made previously[14] that *A. ceratodes* displays a remarkably rich and variable sulfur metabolism.

From the known average blood cell [V(III)] of 99 mM for the S85 sample and 79 mM for the S86 sample,[14] the average sulfate:V(III) concentration ratios are about 1.1:1.0 and 1.9:1.0, respectively. Since the [sulfate]:[V(III)] ratio in sample S85 is less than the 1.5:1.0 ratio of crystalline $V_2(SO_4)_3$, the average blood cell [sulfate] is insufficient to balance the full ionic charge of endogenous vanadium(III). However, inclusion of average [sulfonate] permits calculation of an [ionic sulfur]:[V(III)] ratio, normalized by charge (i.e., $(2[SO_4^{2-}] + [RSO_3^-])/(3[V^{3+}])$). This ratio is 1.1:1.0 in sample S85 and 1.6:1.0 in sample S86. In a stoichiometric complex the charge-balance ratio must be 1:1. Therefore in each case, the combined average blood cell [sulfate] + [sulfonate] is more than sufficient to balance the full ionic charge of endogenous vanadium(III).

A Complexation Model for Blood-Cell Vanadium and Sulfate. The sulfur K-edge XAS spectrum of the S85 sample indicated the *in vivo* complexation of V(III) and sulfate. In contrast, the S86 blood cell sample yielded sulfur K-edge XAS data indicating no interaction between endogenous vanadium(III) and sulfate. The average total blood cell [V(III)] and [sulfate] and the pH of the requisite medium[6,14] are known for each blood cell sample. Therefore the complexiometric constants for vanadium(III) and sulfate of Meier *et al.*[57] and the known K_b of SO_4^{2-} (83.2 M^{-1}) can be used to calculate the average concentrations of all of the endogenous vanadium(III) and sulfate equilomers. This calculational model assumes the colocation of all of the relevant species, a presumption for which there is good evidence in this case[6,14,23] and which is supported more generally;[2,4,11,25,87−89] however, see below.

For the S85 sample, the calculation yields blood cell concentrations of $[SO_4^{2-}]_{free} = 8.5$ mM; $[HSO_4^-] = 79.7$ mM; $[V(III)]_{free} = 77.7$ mM; $[(H_2O)_5VSO_4^+] = 20.9$ mM; and $[(H_2O)_4V(SO_4)_2^-] = 0.45$ mM. The complexed [sulfate] thus calculates to about 92% of the total [sulfate], accounting for the observed strongly broadened sulfur K-edge XAS spectrum.

The sulfur K-edge XAS spectrum of sample S86 exhibited no detectable V(III)−sulfate interaction.[14] Therefore the data support a chemical model which only includes an endogenous protonation equilibrium for the blood cell sulfate. In this case the calculated blood cell $[SO_4^{2-}]_{free} = 41.3$ mM and $[HSO_4^-] = 108.6$ mM, indicating 72% protonation of sulfate. This is comparable to the 72.5% protonated sulfate calculated for the S85 sample.

As mentioned previously,[14] the sulfur K-edge XAS spectrum of the S85 sample was much more strongly broadened than that of the S86 sample. An explicit and independent chemical rationale for this finding is now available in that an additional 20% of the endogenous sulfate is calculated to be bound to V(III) in the S85 blood cells.

The calculated blood cell equilomer concentrations constitute a retrodictive explanation of the observed broadening of sulfur K-edge XAS spectra based upon a simple inorganic model of endogenous vanadium and sulfate. Explicit numerical fits to future blood cell sulfur K-edge XAS spectra using the sulfur K-edge XAS spectra of appropriate inorganic solutions can be used to test this inorganic model. Whether or not the fits are successful will constructively examine the assumption that all of the vanadium, sulfate, and acid are indeed collocated and in solution in any given blood cell sample. That is, other modes of blood cell vanadium or sulfate storage will be implied if fits based upon a simple solution model are confounded.

However, fits to sulfur K-edge XAS spectra are also complicated by the contributions of low-valent sulfur to the total sulfur K-edge XAS spectra of blood cells. Alternatively, intracellular vanadium(III)−sulfate complexes can be observed using vanadium K-edge XAS of whole blood cells.[23] Analogous fitting experiments using the XAS spectra of appropriate vanadium models can therefore provide an independent test of the complexation model described here.

In summary, we have shown that the sulfur species contained within *A. ceratodes* blood cells are almost exclusively cytosolic. Blood cell membranes contain traces of sulfate and low-valent sulfur, but contain no observable lipophilic sulfonic acids or sulfate esters. Blood cell sulfur distributions and concentrations can change radically across a single year. Intrinsic sulfate ion is heavily complexed by protons, or by both protons and vanadium(III), as indicated by broadening of the sulfur K-edge XAS spectra of whole blood cells. This broadening is due to lowering of the tetrahedral symmetry of sulfate to C_{3v} symmetry following complexation. Experiments are in progress to test these results by using vanadium K-edge XAS to examine whole blood cells from the perspective of endogenous vanadium. Extending the XAS approach to ascidians of other species and to specimens of *A. ceratodes* from locales other than Monterey Bay is an ongoing project.

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