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XAS Spectroscopy, Sulfur, and the Brew Within Blood Cells from *Ascidia ceratodes*

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Abstract

We report the first use of K-edge x-ray absorption spectroscopy (XAS) as a direct spectroscopic probe of pH and cytosolic *emf* within living cells. A new accuracy metric of model-based fits to K-edge spectra is further developed. Sulfur functional groups in three collections of living blood cells and one sample of cleared blood plasma from the tunicate *Ascidia ceratodes* were speciated using K-edge XAS. Cysteine and cystine, the preferred thiol-disulfide model, averaged about 12% of total sulfur. Sulfate monoesters and cyclic diesters unexpectedly constituted 36% of blood cell sulfur. Soluble sulfate averaged about 25% across the three blood cell samples, while the ratio of SO_4^{2-} to HSO_4^- implied average signet ring vacuolar pH values of 0.85, 1.4, or 3.1. Intracellular $(\text{VSO}_4)^+$ was unobserved, while $[\text{V}(\text{RSO}_3)_n]^{(3-n)+}$ was detected in the two lowest pH blood cell samples. About 5% of sulfur was distributed as mono- or dibenzothiophene or ethylene-epi-sulfide, or as a thiadiazole reminiscent of the polycarpathamines. Blood plasma was dominated by sulfate (83%), but with 15% of an alkylsulfate ester and about 2% of low-valent sulfur. Gravimetric analysis of soluble sulfate yielded average concentrations of blood cell sulfur. Average [cysteine] and [cystine] (ranging ~10–30 mM and ~20–90 mM, respectively) implied a blood-cell cytosolic *emf* values of approximately –0.20 V. High cellular [cysteine] is consistent with the proposed model for enzymatic reduction of vanadate by endogenous thiol, wherein the trajectory of metal site-symmetry is controlled and directed through to a thermodynamically favored 7-coordinate V(III) product.

Keywords

x-ray absorption; XAS; ascidian; sulfur; speciation; *emf*; blood cells

1. Introduction

Ascidia ceratodes is a filter-feeding marine Urochordate, of order Phlebobranchia [1]. Ascidiaceans have a motile juvenile form complete with notochord, but the adult is sessile and a morphological invertebrate. This somatic transition shows that ascidiaceans lay very close to the evolutionary vertex of vertebrates and invertebrates [2]. Phlebobranch and Aplousobranch

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ascidians actively remove vanadate from sea water and transport it to specialized vacuoles within “signet-ring” cells [3–7]. Within Phlebobranchs, vanadate is reduced to V(III), reaching 0.1 M within the signet ring vacuoles, which also are highly acidic due to endogenous sulfuric acid, possibly reaching pH 0 [7–10]. Fossilized ascidians dating back to the Cambrian show little difference from the modern forms, and can exhibit co-localized enrichment of vanadium [11, 12].

Ascidians also evidence an extraordinary sulfur metabolism [13–22], including aminoacid-derived thiazoles [20], and exotic polythiane alkaloids [20–22], that may promise new pharmaceuticals [23–25]. In the blood cells of *Ascidia ceratodes* [17, 26], and likely also in *A. gemmata* [16], thiol/disulfide, sulfonic acid, and sulfate are present in respective concentrations ranging from tens to hundreds of mM. Recently, the sulfated polysaccharide heparin was isolated from granulocytes of *Styela plicata* [27], joining the known ascidian sulfated galactose polymers [28].

Previous analysis of sulfur K-edge x-ray absorption (XAS) spectra of *A. ceratodes* blood cells found both soluble and membrane-bound forms of sulfur [26]. Sulfur in cleared blood plasma was described as primarily free sulfate with traces of thiol/disulfide. However, all these deductions relied upon empirical Gaussian models of sulfate and sulfonate XAS spectra that were used to fit blood cell XAS.

Greatly improved chemical resolution is now available in sulfur K-edge XAS analysis, through fits using physically relevant XAS spectra of sulfur functional group models [10, 29, 30]. This approach not only permits discrimination between closely related functional groups, e.g., thiol/disulfide or sulfonate/sulfonamide, but also distinguishes the same functional group within different chemical environments, e.g., cyclic from linear disulfides [31, 32]. Relative fractions of each functional group are obtained, while parallel chemical analysis can convert fractions into concentrations.

This study reports detailed speciations of sulfur within three whole blood cell samples from *A. ceratodes* and one cell-free blood plasma sample, using functional group fits to sulfur K-edge XAS spectra. Both milieus revealed unexpected low- and high-valent sulfur functionalities. We also report further development of a new accuracy metric developed to assess speciations using K-edge XAS [33]. The problem of systematic error is addressed, allowing a more quantitative appraisal of fit reliability.

Sulfur fractions and functional groups in Henze solution, in washed cell membranes, and in sulfate-free blood cell lysate will be reported separately. Also described later will be the results of fits to more recent sulfur K-edge XAS measurements of blood cells from *A. ceratodes* of Bodega Bay, California, and further blood cell products from a new collection of Monterey Bay specimens.

2. Materials and Methods

The three *Ascidia ceratodes* whole blood cell collections were previously collected across 18 months from the Monterey Bay Yacht Harbor, Monterey, California, and were designated “S85,” “S86,” and “W87” [17, 26]; a terminology retained here. The original collection dates were, respectively, 1 June 1985, 27 May 1986, and 12 January 1987.

The removal and processing of blood cells and cell-free blood plasma, and the clearance of blood cell surface sulfate were described earlier [17, 26]. Following preparation of the cell-free plasma, no evidence for lysis was observed in the discarded pellet of packed cells. Sulfur K-edge XAS spectra were measured shortly after collection of the specimens.

2.2 Chemical Models and XAS Measurements

Sulfur K-edge XAS spectra of the three blood cell samples and of cell-free plasma were measured at Stanford Synchrotron Radiation Lightsource (SSRL) second-generation (2G) wiggler beam line 6-2 or 2G beam line 4-2 (sample S86) as described previously [17, 26]. Beam line characteristics including detuning are given in the earlier reports, and the specific tests that ensured the absence of harmonic contamination are described in Section 6 of the Supporting Information. Most of the model compound sulfur K-edge XAS spectra were reported earlier [31, 33]. All model compounds were measured at ambient temperature, using a helium x-ray beam path to minimize attenuation, within a dynamically purged sample chamber that also provided an anoxic environment. The compound $K_5[V(\text{oxalate})_2(\text{SO}_4)_2]$ was the kind gift of Prof. Kan Kanamori, Department of Chemistry, University of Toyama, Japan. The compound $[\text{VO}(2,2':6',2''\text{-terpyridine})(\text{O},\text{O}-\text{SO}_4)]\cdot\text{H}_2\text{O}$ was prepared using the published method [34]. The further model compounds described below were purchased from Sigma-Aldrich Chemical Co. and used as received.

The following model XAS spectra were measured on SSRL 2G wiggler beam line 6-2, with a 10 kG wiggler field, about 96 mA ring current, and using a Si(111) monochromator: S-(tert-butylthio)-L-cysteine, finely divided powder on tape; dibenzyltrisulfide, 32 mM solution in p-xylene; (+/-)-6-thioctic acid, 32 mM solution in 250 mM sodium citrate buffer, pH 7.1; glutathiol, 93.6 mM, in degassed anaerobic sodium citrate buffer, pH 7.1; dimethyltartrate-2,3-O-cyclic sulfate (98 mM) and propylene cyclic sulfate (97 mM) as solutions in N,N-dimethylformamide, and; $K_5[V(\text{oxalate})_2(\text{SO}_4)_2]$ and $[\text{VO}(2,2':6',2''\text{-terpy})(\text{O},\text{O}-\text{SO}_4)]\cdot\text{H}_2\text{O}$ as solids finely ground with boron nitride in an agate mortar and pestle.

The XAS spectra of 5,5'-dithio-bis(2-nitrobenzoic acid), 50 mM in pH 7.2 250 mM sodium citrate buffer, and 4,4'-dipyridyldisulfide and diphenyldisulfide, each 50 mM in p-xylene, were measured on 3G SSRL beam line 4-3 using a Si(111) monochromator, at 3 GeV and about 167 mA current.

Preparation of 1.0 M $V(\text{CF}_3\text{COO})_3$ was described previously [8, 26]. Chemical speciation of dissolved aqueous V(III)-sulfate with pH was modeled using the equilibrium constants of Meier, et al. [35], and the Medusa software written by Dr. Ignasi Puigdomenech. Medusa is available as freeware from the KTH Royal Institute of Technology, Valhallavägen: <http://www.kemi.kth.se/medusa/>.

2.3 Normalization of XAS Spectra

Although they had been reported earlier, all the blood cell XAS spectra analyzed here were renormalized using the program PySpline, prior to performing the fits described below. PySpline provides four data windows (baseline, spline, EXAFS, and Fourier transform) that present real-time updates of data processing experiments. It also allows up to 10 spline knot-points that accommodate such slope gradients as may appear in the high-energy portion of the XAS spectrum. Splines were chosen that passed through the rising K-edge at unit intensity to assure that the relative intensities were unchanged; see Figure S1 and Figure S2 in the Supplemental Information. PySpline was written by Dr. Adam Tenderholt, and is available as freeware: <http://sourceforge.net/projects/pyspline/> [36].

Overall tilt in the raw XAS spectrum was first reduced or removed by fitting a polynomial to the pre-edge energy region, and subtracting this polynomial from the entire spectrum. Detailed inspections of the baseline subtraction were made to assure minimal residual tilt near the onset of the low-valent sulfur rising K-edge, which can exert a disproportionate effect on subsequent fits [33]. The baseline-subtracted XAS was then fit over the full energy range using a piece-wise polynomial spline. Finally the entire baseline subtracted XAS was divided by the best-fit spline for normalization to unit intensity. Any very low frequency

curvature, sometimes produced by local monochromator heating, was removed during this step. As noted above, care was taken that the fitted spline should pass along unit intensity throughout the immediate rising K-edge energy region (Figure S1, S2) [33].

2.4 Fits and Error Analysis

Fits were carried out in the piece-wise fashion described previously [33], using the program DATFIT, which is part of the EXAFSPAK software suite written by Prof. Graham George, University of Saskatchewan. EXAFSPAK is available on the SSRL website: <http://www-ssrl.slac.stanford.edu/exafspak.html>. DATFIT permits up to ten model spectra in a fitting experiment.

Fits were carried out piece-wise because the continuum intensity of low valent sulfur extends throughout the x-ray absorption near-edge spectroscopy (XANES) energy region of high-valent sulfur. Likewise, intensity from the high-valent sulfur tails back into the XANES region of low-valent sulfur. This coupling of intensities makes extremely difficult any fit to the full XAS spectrum of a complex multi-valent sulfur sample because changes of model in one sulfur valence region can strongly impact the fit in the other. Under these conditions, even if the new model is correct, the goodness-of-fit parameter may worsen. The experiment is greatly advantaged if each XANES region is fit separately. Experience showed that using a pseudo-Voigt to approximate the high-energy tail in the low valent sulfur XANES region gave much better results than using a linear offset to approximate the low-valent sulfur continuum intensity in the high-valent XANES region. Finally, in the experiments described here, the low- and high-valent sulfur XANES fits required more than five models each, so that a full fit exceeded the DATFIT maximum.

Thus, the initial low-valent sulfur fits were carried out over the 2460–2480 eV energy range. The pseudo-Voigt, added at 2481 eV, was allowed to float in position and intensity. The final fit to low-valent sulfur was then combined into a composite XAS spectrum of normalized intensity, made using the XAS spectra of the models entering the fit but excluding the pseudo-Voigt. This composite XAS of low-valent blood cell sulfur was used as a single entry into the fits to the full blood cell K-edge XAS, carried out over the energy range 2460–2490 eV. The full methodology has been described earlier [33].

The goodness-of-fit variance provided by DATFIT is $\sigma^2 = \sum_i (data - fit)_i^2 / (n - v)$, where “ n ” is the number of data points and “ v ” is the number of fitted variables. This equation was misreported in [33]. For an acceptable fit, the second derivative of the fit was required to closely match the second derivative of the sample XAS spectrum. The first derivatives of XAS spectra were consistently 3-point smoothed, and the second derivatives 5-point further smoothed. Comparison of smoothed with unsmoothed derivative spectra indicated no significant distortion by this procedure. Evaluation of fit candidates always employed identically smoothed derivative spectra.

The total uncertainty of each final fit due to systematic error was calculated as,

$$\pm \sigma_{sys} = \sqrt{(\sigma_{rms})_{lv}^2 + (1 - TF)^2}, \quad 1$$

where $(\sigma_{rms})_{lv} = \sqrt{\sum_i (\sigma_f)_i^2 / n}$, and $\pm \sigma_f$ is the estimated standard deviation (*e.s.d.*) of each of “ n ” fitted low-valent sulfur fractions, f . The TF is Total Fraction, and $TF = \sum_{i=1}^n f_i$ in the

final fit to the XAS spectrum over the full energy range. $TF = 1.00$ in a perfect fit. The root-mean-square (*r.m.s.*) of the *e.s.d.s* of the fitted low-valent fractions represents the average uncertainty carried into the fit of the full XANES spectrum by the composite low-valent sulfur XAS spectrum. As described elsewhere, the value of TF invariably departs from unity because the systematic errors present in individual XAS spectra accumulate within a fit [33]. Departures of TF from unity will also arise if the array of model functional groups is incorrect or incomplete. The combined $\pm\sigma_{sys}$ is a summation of all these inaccuracies.

Systematic error as represented by the departure of TF from unity was unavailable from the fits to low-valent sulfur because the added pseudo-Voigt greatly dominated the total fit intensity. Therefore, the uncertainty in the low-valent fit was propagated as the *r.m.s.* of the *e.s.d.s* as described above. The final-fit fractions were normalized to unit sum and the final $\pm\sigma_{sys}$ is a measure of the fractional accuracy. The uncertainty in the derived vacuolar pH or cytosolic *emf* was propagated as the root-sum-square (*r.s.s.*) of the uncertainties of the fitted fractions used in the calculations [37].

3. Results

3.1 Whole blood cell XAS

Figure 1 shows the sulfur K-edge XAS spectra of the three whole blood cell samples examined in this study. These blood cell spectra were reported previously [17], but have been renormalized and now display somewhat different rising K-edge intensities. The reason for the amendment is that modern processing allows superior removal of slope-change artifacts that can affect normalization (see Section 2.3 in Materials and Methods, Results Section 2.3 below, and Figures S1–S5 in the Supporting Information).

The rising edge XANES of low valent sulfur is visible as the feature at 2473.5 eV, while the high valent sulfur XANES maximizes near 2482.4 eV. Thus, all three blood cell XAS spectra indicate significant concentrations of both low-valent and high valent sulfur. At energies above the bound state (just beyond the XANES features), the ionization probability approaches unity. Sample S86 is unique in that the normalized low-valent ionization intensity near 2476 eV and the high-valent intensity near 2488 eV are nearly equal, implying comparable low- and high-valent sulfur quantities. Distinct differences in the structure and intensity of the high-energy XANES (Figure 1, inset) further imply considerable variability in high-valent sulfur among the samples.

3.2 The XAS Modeling Approach to Blood Cell Sulfur

Model-based fits to the XANES spectra of complex samples take advantage of the unique K-edge XAS features that characterize and distinguish individual sulfur functional groups [29, 31, 38]. The ability to distinguish sulfur functionality using XAS extends even to the same functional group within different structural environments [31, 32]. Thus, for example, cystine is readily distinguishable from glutathione, and benzothiophene from dibenzothiophene. The fits herein should be understood as providing predictions that, within a few ångström units of sulfur, the biogenic sulfur functional groups are structurally similar to the successful models, but include no information about possible more extended structures.

Fits were carried out using a library of K-edge XAS spectra of model sulfur compounds of known functionality. Previous work showed that the low- and high-energy blood cell sulfur features in Figure 1 are broadly consistent with thiol-disulfide and alkylsulfonate-sulfate, respectively [13, 26]. A Raman spectroscopic experiment later confirmed an alkylsulfonate ion within blood cell homogenates from *Ascidia gemmata* [16]. This prior knowledge informed the initial approach to the model-based fits. Thiol-disulfide were modeled as

cysteine-cystine or as glutathiol-glutathione. Glutathiol, GSH, is the thiol redox pair of glutathione, GSSG. These couples are distinguishable in high-resolution sulfur K-edge XAS spectra, and are thus distinguishable within blood cells, given XAS spectra of sufficiently high signal-to-noise (S/N). Elements of a glutathione metabolism have been detected in ascidians [39].

As noted in the Introduction, ascidians are known to deploy an extraordinary array of secondary metabolites, possibly organized for chemical defense [40–42]. This array includes polysulfides, sulfurous alkaloids, and cyclic post-translationally-modified sulfur-containing polypeptides. During the fits to blood cell XAS spectra, attempts were made to detect some of these functional groups by including such model XAS spectra as benzyltrisulfide and bis(tert-dodecyl)pentasulfide. Alternative disulfide models included, but were not limited to, diphenyldisulfide, thiocetic acid, and S-tert-butylthio-L-cysteine. Thiazolium was modeled using thiamine and 3,3-diethylthiacyanine. Aromatic sulfur models included benzothiophene, dibenzothiophene, thionine perchlorate, and thianthrene. Unusual functional groups included thiourea, ethylene-epi-sulfide, 2,1,3-benzothiadiazole, trimethylsulfonium iodide, and 1-(R)(-)-thiocamphor.

The investigation of high-valent sulfur included not only sulfate and alkylsulfonic acid, the latter modeled as methanesulfonate, but also esters of sulfated glycosides and hydrocarbons as found in ascidians [25, 43, 44]. The sulfur K-edge XAS spectra of pendant mono-esters and cyclic diesters are readily distinguishable, and vary strongly with the details of S-O-C bonding and structure (Figure S6 of the Supporting Information).

Intracellular acidity and endogenous V(III) were explored using the sulfur K-edge XAS of dissolved HSO_4^- and SO_4^{2-} , and of the $(\text{VSO}_4)^+$ complex ion, respectively. These were represented as solutions of pH 0.0 and pH 6.3 sulfate, or as V(III) and sulfate in 14:1 ratio solution in pH 1.7 aqueous CF_3COOH . The sulfur K-edge XAS of sulfate systematically broadens on titration with V(III) or acid [17], and the latter effect exactly follows the pK_a of hydrogensulfate [45]. Broadening accompanies the nominal symmetry change of sulfate ion from T_d to C_{3v} during complexation by H^+ or V(III). In pH 0.0 solution, 99% of dissolved sulfate is monoprotonated while, with 14-fold excess V(III), dissolved sulfate in pH 1.7 solution is distributed as ~84% $(\text{VSO}_4)^+$, ~10% HSO_4^- , ~5% SO_4^{2-} and ~1% $(\text{VSO}_4)_2^-$ [35]. The three sulfate solution states -- free, protonated, and V(III)-bound -- are readily distinguished using sulfur K-edge XAS, Figure 2.

It is thus possible to differentiate sulfate among its free, complexed, and protonated states, using sulfur K-edge XAS. All solutions of sulfate between pH 0 and pH 7 can be represented as fractional combinations of the pH 6.3 (~100% SO_4^{2-}) and pH 0.0 (~99% HSO_4^-) forms, allowing an independent estimate of intracellular pH. Likewise the broadened sulfur K-edge XAS spectrum of $(\text{VSO}_4)^+$ tests for an intracellular complex ion. Using XAS spectroscopy, the $(\text{VSO}_4)^+$ ion had previously been detected in frozen blood cell packs of *A. ceratodes* [10, 46, 47], but not in those of *Phallusia nigra* [48].

The sulfur K-edge XAS of dissolved methanesulfonate is virtually insensitive to pH or [V(III)], because CH_3SO_3^- is a very weak Brønsted or Lewis base. However, the sulfur K-edge XAS of 1 M $\text{V}(\text{CH}_3\text{SO}_3)_3$ in 30 mM $\text{CH}_3\text{SO}_3\text{H}$ showed evidence of a $\text{CH}_3\text{SO}_3\text{-V(III)}$ association [26], and was used here to model a direct V(III)-sulfonate interaction.

As noted in the Materials and Methods Section, blood cell sulfur XAS spectra were fit in a piece-wise fashion. Initially, the low-valent sulfur XAS was fit over the range 2460–2480 eV. For these fits, a pseudo-Voigt was added at 2481 eV and allowed to float in position and intensity. This pseudo-Voigt approximated the XAS intensity of high-valent sulfur that tails

back into the lower energy XAS region (cf. Figure 3a). Once a good fit was achieved, a composite XAS spectrum of the low-valent blood cell sulfur was prepared consisting of a linear sum of the composite XAS spectra weighted by their fraction in the fit. The sum of fractions was normalized to 1.00. This combined XAS spectrum represented the complete contribution of low-valent sulfur to the full blood cell XAS spectrum. The composite low valent sulfur XAS spectrum could then be used, along with the XAS spectra of high-valent sulfur models, to fit each full XANES spectrum over the entire 2460–2490 eV energy range. Equations 2 summarize the method,

$$\begin{aligned} \text{Low-valent fit} &= X_{lv} = \sum_i w_i \chi_i \text{ and } \pm \sigma_{lv} = \sqrt{\sum_i (w_i \sigma_i)^2 / n - 1} \\ \text{Full fit} &= X_{full} = w_j X_{lv} + \sum_k w_k \chi_k \text{ and } \pm \sigma_{total} = \sqrt{(w_j \sigma_{lv})^2 + \sigma_{full}^2}, \end{aligned} \quad 2$$

where $w_{i,k}$ is the fractional weight of each $\chi_{i,k}$ of the “n” model XAS spectra. The $\pm \sigma_{lv}$ is the total uncertainty in X_{lv} , which is carried forward into the uncertainty estimate for the fit to the full XANES, and the $\pm \sigma_i$ are the *e.s.d.s* in the low-valent fit fractions. Finally,

$\pm \sigma_{full} = \sqrt{(1 - TF)^2}$ is the systematic error in the final fit over the full energy range (see equation 1 in the Materials and Methods section), and $\pm \sigma_{total}$ is the final uncertainty represented by the *r.s.s.* of $\pm \sigma_{lv}$ and $\pm \sigma_{full}$. The $\pm \sigma_{full}$ invariably dominated $\pm \sigma_{total}$.

3.3 Fits to Blood Cell Sulfur K-edge XAS

Figure 3 shows the individual low-valent and high-valent fits to the sulfur K-edge XAS of the S86 whole blood cell sample, along with the model XAS spectra used in the fits. Both low- and high-valent sulfur proved to be complex mixtures. The fits generally confirmed the original finding that thiol-disulfide and alkylsulfonate-sulfate are the major low- and high-valent forms of *A. ceratodes* blood cell sulfur [13, 49–52]. However, the complete reality proved much more interesting.

For low-valent sulfur, all three blood cell samples were slightly better fit using the redox pair cysteine/cystine rather than glutathiol/glutathione. Although fits utilizing the latter were nearly as good, e.g., for sample S85, $\sigma^2 = 0.41 \times 10^{-4}$ vs. 0.47×10^{-4} , respectively, the small preference for the cysteine redox pair persisted throughout. Nevertheless, in all cases, the signal-to-noise (S/N) was insufficient to fully exclude the glutathione pair. Additional disulfides, such as thiocetic acid, diphenyldisulfide, and di-tertbutyldisulfide, were invariably rejected by the fits.

Significant fractions of aromatic sulfur were found, represented by benzo- or dibenzothiophene, or thianthrene. Preference was exclusive to one or another model; never a mixture. Alternative aromatic functionality such as thionine, thiamine, or thiacyanine were invariably excluded. The ethylene epi-sulfide model represented 8% of total sulfur in blood cell sample S86. Linear polysulfide models, as well as elemental sulfur itself, were invariably excluded. Finally 5% of the 2,1,3-benzothiadiazole model was needed to fit the XAS of blood cell sample S86. This model implies an alkaloid with =N-S-N= functionality similar to the polycarpathamines recently discovered in *Polycarpa aurata* [53].

In Figure 4 are the second derivative XAS spectra of sample S86 and the fit. Although only two major high-valent features are visible, they represent a far more complex mixture than the originally hypothesized sulfate plus sulfonate, alone. The low-valent sulfur XAS displays the double-peak reminiscent of disulfides, but the fit again indicated a much more complex reality. Analogous complexity emerged in the fits for all three blood cell samples, which are shown in Figure S7, Figure S8, and Figure S9 in the Supporting Information. The

XAS of samples S85 and W87 were of higher S/N and the systematic error of their final fits was 4–10× smaller than that for sample S86 (Table 1, below).

Finally, Figure 5a shows the sulfur K-edge XAS spectrum and the fit of the cleared blood plasma sample. Figure 5b presents the second derivatives. The plasma originated from the S86 collection of *A. ceratodes*, but did not come from the same specimens as the assessed blood cells.

The centrifuged plasma sample was colorless and transparent, rather than opalescent as reported for *P. stolonifera* [54], or yellow [55]. Although the second derivative of the high-valent sulfur XAS shows only one feature with no obvious shoulders, the best fits required two components. The majority intensity was fit with the XAS of pH 6.3 sulfate, as expected. Surprisingly, the best fit ($\sigma^2 = 0.51 \times 10^{-2}$) included the XAS of pH 0.0 sulfate as the second component. A fit that included the XAS of the methylsulfate anion as the second component was slightly worse ($\sigma^2 = 0.73 \times 10^{-2}$). Myo-inositol hexasulfate as the second component gave a poorer result ($\sigma^2 = 0.88 \times 10^{-2}$), as did methylsulfonate ($\sigma^2 = 0.99 \times 10^{-2}$). Neutral sulfate alone yielded $\sigma^2 = 1.58 \times 10^{-2}$. Glutathione was used to model the evident trace of low-valent sulfur at 2473 eV (Figure 5a, inset), because blood plasma is oxidic. The XAS of cystine could as well have been used.

In the best fit, the $\text{SO}_4^{2-}:\text{HSO}_4^- = 0.73:0.25$ ratio implied a plasma pH = 2.4. However, cleared *A. ceratodes* blood plasma was invariably pH = 6.7. No evidence of cell lysis was observed during preparation of the plasma sample, obviating an artifactual acidity from this source.

A hypothesis rationalizing this best-fit result is that *A. ceratodes* blood plasma may include a soluble hydrogensulfate porter in large concentration (25% of plasma $[\text{SO}_4^{2-}] \approx 5$ mM [26]). Although no ascidian sulfate or hydrogensulfate plasma porter is known, a 34.6 kD bacterial sulfate porter provides a single well-buried binding site, and over the range pH 5–9 meets the requirement of isolation from solution pH equilibration [56, 57]. With this protein as a model, the fit-derived 5 mM hydrogensulfate concentration implies 173 mg/mL of plasma protein. However, the concentration of plasma proteins in ascidians is known to be very low (~0.3 mg/mL; 1 mg/mL) [58, 59], including 1.09 mg/mL in *A. ceratodes* [60]. The very weak intensity of the 2473.5 eV XAS feature (Figure 5a, inset) is consistent with the low *A. ceratodes* plasma protein concentration. These protein concentrations are more than two orders of magnitude lower than the plasma porter concentration required by the fit constraint. Alternatively, if the entire 1.09 mg/mL of *A. ceratodes* plasma protein is assigned to a sulfate porter, the fit-derived 5 mM concentration implies an unlikely 220 D porter. This formula weight is less than that of lysyl-lysine, and implies so small a molecule as to be unlikely able to isolate hydrogensulfate from plasma pH equilibration.

All these considerations taken together eliminate the likelihood of a soluble plasma hydrogensulfate porter in *A. ceratodes*. Thus the hydrogensulfate fit was rejected as biophysically implausible. Nevertheless, it remains problematic that the hydrogensulfate model produced the better fit. We can only suggest empirical happenstance, and that a better alkyl sulfate XAS model may resolve this issue.

3.4 The Sulfur Chemistry of *A. ceratodes* Whole Blood

Table 1 provides a list of the sulfur models used, along with the fractional amounts of each found in the fits to the blood cell and plasma K-edge XAS spectra.

In Table 1, the $\pm\sigma_{\text{sys}}$ row represents the average percent systematic uncertainty in the fitted fractions; e.g., for S86 L-cysteine = 0.04 ± 0.01 .

The structures of the several forms of sulfur found by the fits are presented in Scheme 1. A list of the models tested and rejected is provided in the Supporting Information.

It is now generally understood that in *Phlebobranch* ascidians, the sulfuric acid [8, 51, 61], and the reduced vanadium [8, 17, 50, 61–63], reside in the large vacuole of signet ring blood cells [5, 42, 64, 65]. These cells are also the origin of the acidity that appears in Henze solution. Assuming that vacuolar sulfate and hydrogensulfate dominate total blood cell sulfate, their fractions report the average vacuolar pH. Thus,

$$\text{Vacuolar pH} = -\log[H^+]_{\text{vacuole}} = -\log \frac{K_a [HSO_4^-]_{\text{vacuole}}}{[SO_4^{2-}]_{\text{vacuole}}}, \quad 3$$

where $[HSO_4^-]$ and $[SO_4^{2-}]$ can be represented by their fitted fractions.

By the same token, assuming they are dominantly cytosolic, the fractions of cysteine and cystine report the average intracellular *emf* as equation 4:

$$2 \text{ Cys-SH} \rightleftharpoons \text{CysS-SCys} + 2H^+ + 2e^- \text{ and } K_{\text{halfcell}} = \frac{[\text{CysS-SCys}][H^+]^2}{[\text{Cys-SH}]^2} \quad 4$$

where K_{halfcell} is the equilibrium constant for the cysteine half of the electrochemical system governing the cytosolic *emf* of the blood cell. The other half of the electrochemical system is probably regulated by the flux of vanadate [66]. As the $[\text{Cys-SH}]$ term is squared in eqn. 4, the Nernst equation for the average intracellular *emf* requires the true concentrations of cysteine and cystine [67], equation 5:

$$E_{\text{cell}} = E^{0'} + \frac{0.059}{2} \log \frac{[\text{CysS-SCys}]}{[\text{Cys-SH}]^2}, \quad 5$$

where E_{cell} is the *emf* of cysteine half-cell, and $E^{0'}$ is the pH 7 standard redox potential of the cysteine-cystine couple. The cytosolic pH of ascidian blood cells has been reliably reported as near neutrality [63], allowing the pH-uncompensated Nernst equation 5 and $E^{0'} = -0.254 \text{ V}$ for the cysteine-cystine couple [68].

The average concentrations of soluble sulfate in blood cell samples S85 and S86 were previously measured to be $110 \pm 3 \text{ mM}$ and $150 \pm 5 \text{ mM}$, respectively [26]. Knowledge of these concentrations plus the fit fractions in Table 1 allows the average intracellular concentrations of the remaining sulfur species to be calculated.

The newly discerned presence of sulfate esters makes evaluation of the original sulfate analysis more complex than initially recognized, however. Total sulfate was estimated as solid BaSO_4 , precipitated from supernatants derived from washed and cleared whole blood cell homogenates [26]. At the time these experiments were done, sulfate esters were not known to be present. Reliability of the analyses for soluble sulfate necessitates the non-hydrolysis of sulfate esters during the Ba^{2+} precipitation step.

Hydrolysis of sulfate esters requires an elevated temperature (ca 100 C) even in strongly acidic solution [69, 70], and Ba^{2+} does not precipitate the sulfate from sulfate esters without prior hot hydrolysis [71]. The estimates of average soluble blood cell $[SO_4^{2-}]$ were carried out at room temperature in pH 2 solution, and the BaSO_4 precipitate was removed immediately by centrifugation. This procedure should have precipitated dissolved sulfate

with negligible hydrolysis of any sulfate esters present. Further Ba^{2+} added to the secondary supernatant produced no further precipitate.

Table 2 includes the estimates of soluble [sulfate], and the calculated whole blood cell [cysteine], [cystine], cytoplasmic *emf*, and vacuolar pH for the three samples.

These calculations again assume that the cysteine and cystine occur in the neutral cell cytosol, and that the sulfate and hydrogensulfate are predominantly in the signet ring vacuoles. The concentrations are averages over the whole cell packs, and no attempt has been made to scale by a ratio of vacuolar to cellular volumes. The average soluble [sulfate] was not determined for sample W87, but for this analysis is assumed to be comparable to the other two blood cell samples. Following this assumption, calculation of W87 sulfur amino acid concentrations and cytoplasmic *emf* produced values equivalent to those of samples S85 and S86.

4. Discussion

Of the several surprises emerging from this work, perhaps the two greatest are the discovery of blood cell sulfate esters, and the complete lack of any evidence for an intracellular $(\text{VSO}_4)^+$ complex ion. The latter result forces a re-evaluation of prior conclusions (see section 4.2 below). The general complexity of sulfur secondary metabolites was also unexpected. A variety of cytotoxic alkylsulfate monoesters have been isolated from both Pacific and Mediterranean ascidians, which have received attention as drug candidates [25]. The sulfone functionality present in *A. conicum* prenyl quinones [25], was not detected here.

4.1 Sulfate esters

Sulfate esters average about a third of the total blood cell sulfur, and represent a significant metabolic investment. The inositol sulfate ester model in blood cell sample S86 implies a carbohydrate linkage. *Styela plicata* is reported to have a circulating sulfated galactoglucan polysaccharide and heparin within granulocytes [27]. Sulfated polysaccharides are also present in the bodies of other ascidians, including *Phallusia nigra* [72, 73].

The alkanesulfate model, methylsulfate, was rejected in the fits to all three blood cell samples. However, cyclic sulfate diester functionalities were found liberally distributed throughout. It thus seems likely that blood cell alkylsulfates in *A. ceratodes* are distinct from the known ascidian alkyl monosulfates [74, 75].

On the other hand, while the carbohydrate ester model *myo*-inositol hexasulfate was excluded from cell-free blood plasma, the methylsulfate ester could be included. This sulfate ester represents 15% of plasma sulfate (~ 3 mM) [26], and may represent one of the known ascidian cytotoxic alkylsulfates [25, 44]. The alkyl ester 1-(12-hydroxy)octadecanyl sulfate was isolated from whole body extracts of an unknown species of *Sidneioides* [76], but whether it originated in blood cells or tissue is unknown. Likewise, the cyclodidemniserinol trisulfate isolated from *Didemnum guttatum* [77], and the cytotoxic alkyl sulfates isolated from *Sidnyum turbinatum* and *A. mentula* [74, 75], are from whole body extracts. Such alkylsulfates, especially the complex alkyl disulfates isolated from the closely related *A. mentula* [74], are candidates for the circulating plasma alkylsulfate in *A. ceratodes*.

Cyclic diesters have not been reported among the ascidian sulfate esters. The carbon frameworks of the sulfate diester XAS model compounds (mannitol, tartrate, etc., Table 1), should not be seen as predictions of specific carbon-backbone structures. However, the Scheme 1 configurations of the sulfate diesters themselves, cis- or trans-pentacyclic, or unstrained (hexacyclic), represent predictions of general sulfate ester configurations. These

sulfate esters may be part of the ascidian immune system [78]. At this point, palatability studies appear to be absent and it is apparently not known whether sulfate esters will deter predation.

No studies indicate whether the dissolved vacuolar sulfate dianion is a source for ester-bound sulfate. Further, given the strong Brønsted acidity and weak Lewis basicity of sulfate esters, these groups are not likely to be protonated in solutions of low pH, or to be complexed by V(III). Sulfur K-edge XAS therefore cannot distinguish whether sulfate monoesters are within the signet ring cell vacuole or in a neutral metal-free cytoplasm.

4.2 Intracellular V(III) and sulfate

Intracellular vanadium-sulfate interactions were originally inferred from proton NMR measurements on living *A. ceratodes* blood cells [50], and from the results of vanadyl ion EPR measurements on frozen blood cell packs [8, 9]. A direct intracellular V(III)-sulfate interaction was later deduced from the broadened, model-validated, Gaussians required to fit the sulfur K-edge XAS spectra of *A. ceratodes* blood cells [17, 26]. Likewise, fits to vanadium K-edge XAS spectra of frozen blood cells, using the vanadium K-edge XAS spectra of discrete model solutions, yielded definitive evidence for endogenous $(\text{VSO}_4)^+$ ion [10, 45, 47].

However, the prior decision for intracellular $(\text{VSO}_4)^+$ complex ion is not applicable to living ambient temperature *A. ceratodes* blood cells. The present work shows that sulfate esters contribute strong broadening to blood cell sulfur XAS spectra in the same energy region as both HSO_4^- and $(\text{VSO}_4)^+$ (Figure 2 and Figure S6). It is now evident that the extra width of the high-energy blood cell sulfur K-edge XAS feature is due to sulfate esters and not from sulfate ion complexed to V(III). The results reported here make our previous conclusion untenable. A clear tactical lesson of the present work, therefore, is that Gaussian fits to XAS spectra do not permit structural specifications.

Figure 6 below models what would be the average solution concentrations of blood cell sulfate and its complex ions, assuming that sulfate and V(III) are collocated within the signet ring vacuole under standard liquid aqueous solution. Under these conditions, the fraction of V(III)-bound sulfate in blood cell samples S85, S86, and W87 would have been 0.13, 0.26, and 0.22, respectively (dashed vertical lines). These levels of $(\text{VSO}_4)_n^{(3-2n)+}$ would have been readily detectable as broadened sulfur K-edge XAS spectra. However repeated fitting experiments invariably rejected the explicitly $(\text{VSO}_4)^+$ XAS model, whereas the pure HSO_4^- model was just as invariably retained.

A clear disparity of results has thus emerged between the sulfur K-edge results reported herein (no detectable intracellular $[\text{VSO}_4]^+$), and the prior fits to the vanadium K-edge XAS spectra (copious intracellular $[\text{VSO}_4]^+$).

In retrospect, a resolution of this disparity is available within the different experimental conditions. The sulfur K-edges were measured at ambient temperature with living blood cells suspended in aqueous sulfate-free isotonic buffered synthetic plasma. In contrast, the vanadium K-edges represented frozen blood cell packs measured at 10 K. The frozen conditions were chosen, in part, because V(III) in acidic room temperature solution was observed to progressively photo-oxidize to VO^{2+} during K-edge XAS measurement. The fragility of living blood cells discouraged attempts to render them anaerobic.

EPR experiments have shown that compositional artifacts are produced on freezing aqueous solutions of transition metals [8, 79, 80]. Freezing was also observed to change the pK_a values governing at least the first deprotonations of $\text{V}(\text{H}_2\text{O})_6^{3+}$ in water solution [45], and to

reorganize the electron distribution among the three copper sites in partially reduced T₂D *Rhus* laccase [81]. Zone-migration and the growth of ice crystals during freezing can increase solute concentrations and the extent of metal ion complexation prior to immobilization. Equilibrium constants can change with temperature, leading to reorganization of complex ions or of redox partitioning during freezing.

We suggest, therefore, that the divergent XAS results with respect to intracellular (VSO₄)⁺ indicate a strong difference between the internal composition of living blood cells at ambient temperature and of frozen blood cells at 10 K. In the vanadium XAS measurements, freezing *A. ceratodes* blood cells evidently induced the association of V(III) and sulfate. Interestingly, in a similar vanadium K-edge measurement, frozen blood cells of *P. nigra* did not yield evidence of vanadium-sulfate complex ions [48]. *P. nigra* and *A. ceratodes* thus apparently segregated intracellular vanadium and sulfate disparately.

The final extent of the V(III)-sulfate association in the frozen solutions and *A. ceratodes* blood cells should have evolved from the distributions within the initially liquid matrix [45]. The fits to the vanadium K-edge XAS of frozen blood cells employed the XAS of frozen V(III)-sulfate model solutions, which are known to reflect liquid-state (VSO₄)⁺ [10, 47]. In the vanadium K-edge fits to frozen blood cells, model solutions without the (VSO₄)⁺ signature were tested and were rejected by the algorithm. Therefore, there is little doubt that (VSO₄)⁺ was present in frozen *A. ceratodes* blood cells at 10 K. However, there is also now little doubt that this complex ion is not present in the living cells at ambient temperature.

Therefore, it is no longer tenable to extrapolate the (VSO₄)⁺ complex ion in frozen blood cells to living blood cells at ambient temperature. It is the ambient temperature result that more certainly represents the biological chemistry of living *A. ceratodes* blood cells and their state in the wild.

However, the (VSO₄)⁺ ion could not have appeared in the frozen cells unless the sulfate and V(III) were mutually accessible by migration. Therefore, the vanadium K-edge fitting results remain reliable in the sense that they demonstrate that the V(III) and sulfate must be proximate within living *A. ceratodes* blood cells, even though not associated. The vanadium results also reflect the extent of association of sulfate and V(III) as it resulted within the frozen vacuole of the signet ring cells. Within *P. nigra* blood cells, resident sulfate and vanadium must be more securely sequestered [48].

In further contrast, a V(III)-alkylsulfonate association was detected within the living blood cells of samples S85 and W87. Comparing the fit using the associational CH₃SO₃-V(III) model to that with CH₃SO₃⁻ alone, for example, yielded respective goodness-of-fit σ^2 values for sample S85 of 3.2×10^{-3} vs. 3.7×10^{-2} and for W87 of 3.5×10^{-3} vs. 1.5×10^{-2} . These differences are statistically definitive.

The fit to the XAS of sample S86 rejected the CH₃SO₃-V(III) model in favor of the free CH₃SO₃⁻ anion ($\sigma^2 = 6.7 \times 10^{-2}$ vs. 3.7×10^{-2} , respectively), in contrast to samples S85 and W87. This difference is correlated with intracellular pH (Table 2), in that the CH₃SO₃-V(III) model was associated with pH < 1.5.

These results are surprising because, under all conditions of solution pH, an alkylsulfonate is a poorer ligand for transition metals than sulfate. For example, titration with V(III) ion produced almost no effect on the sulfur K-edge XAS of aqueous pH 1.8 methanesulfonate, even to 10-fold stoichiometric V(III) excess [17, 26]. Therefore, the association of CH₃SO₃⁻ and V(III) in solution is not expected, and their apparent association within blood cells requires an explanation.

Previous micro-XANES spectra at the vanadium K-edge have indicated a vanadium-rich granule in signet-ring cells from *A. sydneiensis samea* [82]. Although the granule vanadium included a significant fraction of VO^{2+} , it seems possible that the $\text{CH}_3\text{SO}_3\text{-V(III)}$ model may reflect this granular vanadium. If so, then the existence of the granule may be pH-dependent.

Figure 6 and Figure S10 indicate that at pH 3.1, most V(aq)^{3+} will be sequestered within hydrolytic oxo-bridged multinuclear complexes, and unavailable to sulfate or alkylsulfonate ligands. The pH-dependent appearance and disappearance of $\text{V(III)-alkylsulfonate}$ within blood cells then implies an acidity-dependant V(III) biological chemistry.

Ascidian blood cells are known to enter an activated state when stressed, which is accompanied by changes in thiol content [83], (see below). It seems possible that variation in intracellular pH and mobilization of V(III) may accompany that state.

4.3 Thiol-disulfide and the mechanism of vanadate reduction

For biochemical reasons discussed below, the blood cell cysteine and cystine deduced from the fits are assumed to reside in the cytosol rather than in the acidic vacuole. The intracellular thiol concentrations are about an order of magnitude greater than the amount of glutathione measured in blood cell homogenates from *A. sydneiensis samea* [84], but this difference may reflect the more concentrated conditions of intact blood cells.

The concentrations of cysteine and cystine imply a mean blood cell cytosolic *emf* of about -0.20 V (Table 2), which is apparently on the positive side of the range for healthy quiescent cells [67, 85]. However, immune-activated ascidian blood cells appear to increase intracellular *emf* by altering the concentration of cytosolic thiols [83]. It seems reasonable that blood cells removed by the usual methods of heart puncture or test incision should enter such a state.

Thiols such as L-cysteine or L-glutathiol remain the best reaction candidates for the biological reduction of V_2O_5 to VO^{2+} or V(III) by ascidians [66, 86]. Likewise, the uniformly high intracellular concentration of cysteine is consistent with participation in this reduction [66]. While neither cysteine nor glutathione alone are able to reduce aqueous VO^{2+} between pH 2–9 [87], reduction becomes facile when a 7-coordinate V(III) product is available [66, 86]. This arises because the reaction channel to a 7-coordinate product imparts an extra 500 mV to the reductive *emf* of VO^{2+} [88]. Therefore, thiol-based biological reduction of vanadate will be facilitated by an enzyme that can direct the reaction trajectory to a seven-coordinate V(III) product. This proposed mechanism calls upon the known inorganic chemistry of vanadium [66, 86], that has direct structural and reactivity cognates in ascidian blood cells [48, 66]. Taken together these considerations render unlikely any prospective reduction model featuring an enzyme thiol active site or a protein rich in backbone cysteines [84].

As the reductive driving force of dissolved thiols increases with pH, it is difficult to conceive that a vanadium reductase operates under the typical pH 1.8 conditions of the signet ring vacuole. The cellular venue for enzymatic vanadate reduction is instead most likely the neutral cytosol. The most parsimonious enzymatic reduction mechanism must include that all the vanadium intermediates remain bound at the active site. Reduction to V(III) should then be followed by transport across the cytoplasmic-vacuolar inter-membrane into the acidic vacuolar interior. This implies the existence of a membrane vanadium transporter. Ockham's Razor allows that the reductase and the membrane transporter be initially hypothesized as the same protein [89, 90]. That is, the proposed vanadate reductase and V(III) membrane transporter may be the same membrane protein, taking up V(V) in the

cytoplasm and releasing V(III) into the vacuole. The release of V(III) could be driven by uptake of vacuolar protons [66].

5. Conclusions

This study has forced a re-evaluation of our previous conclusions concerning the intracellular presence of the $(\text{VSO}_4)^+$ complex ion in *A. ceratodes* blood cells [10, 47]. All results to this date indicate the co-location of sulfate and V(III) within the native signet ring vacuole, but the $(\text{VSO}_4)^+$ complex ion is now seen as absent in the living blood cell. Only a V(III)-alkylsulfonate association was detected, thus far only within blood cells also of intracellular $\text{pH} < 1.5$, and which may be granular. Thiol and disulfide exhibit the high concentrations consistent with the mechanism of enzymatic reduction proposed as the source of the copious Phlebobranch blood cell V(III) [66]. It also seems possible that whole blood cell studies may report a state not typically present in an undisturbed ascidian.

Clearly, in answering some questions this study has opened the door to many others. The chemistry and biochemistry of ascidian blood cells remains a field rich with opportunity for new discoveries. Finally, this study represents the first use of XAS as a direct spectroscopic probe to determine the pH and cytosolic *emf* within living cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Sulfur in living blood cells of *A. ceratodes* is speciated using K-edge x-ray absorption spectroscopy (XAS).
- The first use of XAS to determine cytosolic emf of living cells.
- The first use of XAS to determine intra-vacuolar pH of living cells.
- A new accuracy metric for XAS spectroscopic fits is developed.
- Basic reappraisal of V(III)-sulfur bioinorganic chemistry in ascidian blood cells.

Figure 1. Sulfur K-edge XAS spectra of whole blood cell packs from *Ascidia ceratodes*, sequentially collected across 18 months: (—) S85; (---) S86, and (....) W87. Inset: expansion of the high-valent sulfur XAS energy region.

Figure 2. Normalized sulfur K-edge XAS spectra of dissolved aqueous sulfate ion under conditions of: (—), pH 6.3; (- -), pH 0.0 CF₃COOH, and; (····), pH 1.7 CF₃COOH with 14×V(CF₃COO)₃, (84% VSO₄⁺). [S]_{total} = 67 mM.

Figure 3.

(-o-), Sulfur K-edge XAS spectrum of whole blood cell sample S86, and; (dark blue), the fit to the XAS. Panel a, low-valent sulfur: (brown), L-cysteine; (red), L-cystine; (aqua blue), 2,1,3-benzothiadiazole; (light green), dibenzothiophene; (purple), ethylene-epi-sulfide, and; (dark green), the pseudo-Voigt (see text). Panel b, high-valent sulfur: (purple), sulfate, pH 6.3; (aqua blue), sulfate, pH 0.0; (dark red), methanesulfonate; (black), 1,3-propylenesulfate; (dark green), 1,2:5,6-Di-O-isopropylidene-D-mannitol-3,4-O-sulfate, and; (light green), *myo*-inositol hexasulfate. The dashed brown line is the total continuum intensity of the low valent sulfur XAS.

Figure 4. Second derivative of: (o), the sulfur K-edge XAS spectrum blood cell sample S86 and; (—), the fit to the XAS.

Figure 5.

a. (o), Sulfur K-edge XAS of cleared blood plasma from *A. ceratodes* and; (—), the fit to the XAS. The components are: (····), sulfate, pH 6.3, and; (- -), methylsulfate. Inset: (o), the low-valent energy region, and; (—), the glutathione model. b. (o), the second derivative of the XAS, and; (—), of the fit.

Figure 6.

The solution behavior of sulfate if co-located with V(III) within a signet ring vacuole under conditions of free diffusion and no competition [35]. Modeled $[\text{V(III)}]$ and $[\text{SO}_4^{2-}]$ are 80 mM and 130 mM, respectively [17, 26]. At $\text{pH} > 2.5$, hydrolysis of V(III) (shown in Figure S10 of the Supporting Information) increasingly displaces sulfate from the complex ions. The dashed vertical lines mark the modeled pH 0.85, 1.4 and 3.1 of blood cell samples S85, S86, and W87, respectively.

Scheme 1.
 Blood Cell Sulfur Functional Groups for *Ascidia ceratodes*

Table 1Functional Group Models for Blood Cell Sulfur in *A. ceratodes*

Sulfur Model	S85	S86	W87	Plasma (S86)
L-cysteine	0.04	0.04	0.03	---
L-cystine	0.05	0.13	0.06	---
Glutathione	---	---	---	0.02
Ethylene episulfide	---	0.08	0.006	---
Benzothiophene	---	---	0.006	---
Dibenzothiophene	---	0.07	---	---
Thianthrene	0.01	---	---	---
2,1,3-benzothiadiazole	---	0.05	---	---
Cysteinesulfinic acid	0.003	---	0.002	---
CH ₃ SO ₃ ⁻	---	0.14	---	---
[V(III)-CH ₃ SO ₃]	0.28	---	0.18	---
propylene-1,3-O-sulfate	0.21	0.10	0.11	---
Mannitol-3,4-O-sulfate	0.16	0.12	0.17	---
Me ₂ Tartrate-2,3-O-sulfate	0.05	---	0.11	---
myo-Inositol hexasulfate	---	0.05	---	---
Methylsulfate	---	---	---	0.15
Sulfate, pH 0.0	0.19	0.015	0.26	---
Sulfate, pH 6.3	0.016	0.208	0.079	0.83
±σ _{sys} (%)	6	24	2	8
S/N	834	232	1180	222

Fractions may not sum to 1.00 due to round-off error. S/N = (1/r.m.s.)_{noise} × white line intensity.

Table 2

Calculated Vacuolar pH and Cytoplasmic EMF

	S85	S86	W87
[Sulfate] _{soluble} (mM)	110±3	150±5	(130) ^a
[Cys-SH] (mM) ^b	21±1	27±6	11±1
[CysS-SCys] (mM) ^b	27±2	87±21	23±1
E _{cytoplasm} (V) ^b	-0.20±0.02	-0.19±0.08	-0.19±0.02
pH ^{c,d}	0.85±0.04	3.1±0.3	1.40±0.01

^a [SO₄²⁻]_{soluble} in W87 is estimated as the average of S85 and S86.

^b The uncertainties are the r.s.s. of the fit $\pm\sigma_{\text{sys}}$ values plus the analytical error in [SO₄²⁻]_{soluble}.

^c uncertainty is the r.s.s. of the fitted sulfate and hydrogensulfate fractions.

^d Previous vanadyl ion EPR measurements yielded pH 1.8 and 2.1, respectively, for blood cell samples from two single-animals of the W87 collection [17].