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A Large Reservoir of Sulfate and Sulfonate Resides within Plasma Cells from *Ascidia ceratodes*, Revealed by X-ray Absorption Near-Edge Structure Spectroscopy[⊥,†]

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ABSTRACT: The study of sulfur within the plasma cells of *Ascidia ceratodes* [Carlson, R. M. K. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2217–2221; Frank, P., Carlson, R. M. K., & Hodgson, K. O. (1986) *Inorg. Chem. 25*, 470–478; Hedman, B., Frank, P., Penner-Hahn, J. E., Roe, A. L., Hodgson, K. O., Carlson, R. M. K., Brown, G., Cerino, J., Hettel, R., Troxel, T., Winick, H., & Yang, J. (1986) *Nucl. Instrum. Methods Phys. Res., Sect. A 246*, 797–800] has been extended with X-ray absorption near-edge structure (XANES) spectroscopy. An intense absorption feature at 2482.4 eV and a second feature at 2473.7 eV indicate a large endogenous sulfate concentration, as well as smaller though significant amounts of thiol or thioether sulfur, respectively. A strong shoulder was observed at 2481.7 eV on the low-energy side of the sulfate absorption edge, deriving from a novel type of sulfur having a slightly lower oxidation state than sulfate sulfur. The line width of the primary transition on the sulfur edge of a vanadium(III) sulfate solution was found to be broadened relative to that of sodium sulfate, possibly deriving from the formation of the VSO_4^+ complex ion [Britton, H. T. S., & Welford, G. (1940) *J. Chem. Soc.*, 761–764; Duffy, J. A., & Macdonald, W. J. D. (1970) *J. Chem. Soc.*, 977–980; Kimura, T., Morinaga, M., & Nakano, J. (1972) *Nippon Kagaku Zaishi*, 664–667]. Similar broadening appears to characterize the oxidized sulfur types in vanadocytes. A very good linear correlation between oxidation state and peak position (in electronvolts) was found for a series of related sulfur compounds. This correlation was used to determine a 5+ oxidation state for the additional sulfur type at 2481.7 eV. Construction of a cellular sulfur minus vanadium(III) sulfate difference spectrum, along with comparison with spectra of known compounds, identified the novel sulfur(V) as an aliphatic sulfonic acid analogous to cysteic acid. The overall sulfonic acid concentration is comparable to that of sulfate in plasma cells and appears to be unprecedented in marine organisms.

The biological chemistry of ascidians (mostly sessile, strictly marine urochordate filter feeders) has consistently displayed highly unusual attributes (Stroeker, 1980; Krishnan, 1975; Smith & Dehnel, 1971; Swinehart et al., 1974; Bruening et al., 1985; Frank et al., 1986). For *Ascidia ceratodes*, significant attention has focused on the inorganic biochemistry of vanadocytes (~60% of the plasma cell population (Biggs & Swinehart, 1979)). Within a related series of organisms, these cells are known to contain within vacuoles (vanadophores) ~1.4 M vanadium(III) (Swinehart et al., 1974; Carlson, 1975; Danskin, 1978; Tullius et al., 1980; Frank et al., 1986[1]) associated with considerable sulfur (Carlson, 1975; Bell et al., 1982; Rowley, 1982; Pirie & Bell, 1984; Frank et al., 1986) and tunichrome (Bruening et al., 1985) (~1 M): a highly modified tripodal trimer of 3,5-dihydroxytyrosine. Small amounts of vanadyl ion are also known to be present

⊥ Dedicated to the memory of Dr. Donald P. Abbott.

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[1] In this work, the A_0 from the EPR spectrum of vanadyl ion within vanadocytes from *Ascidia nigra*, as presented in Dingley et al. (1981), was calculated and reported by us to be 1.06×10^{-2} cm^{-1}. However, the correct value is 1.01×10^{-2} cm^{-1}, indicative that a weak carboxylate-like complex of vanadyl ion predominates in this organism (Reeder & Rieger, 1971; Chasteen, 1981). The width of the $(-^7/_2)_\parallel$ line in the frozen-solution EPR spectrum arising from these cells is, therefore, not a direct measure of pH (Frank et al., 1986). We note, however, that vanadyl carboxylate complexes are stable to very low pH values (Kustin & Pizer, 1970; Reeder & Rieger, 1971) and appear to have been detected by EPR in vanadocytes from *Ascidia mentula* (Bell et al., 1982).

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in ascidian vanadocytes (Tullius et al., 1980; Dingley et al., 1982; Bell et al., 1982; Frank et al., 1986). When present as the pentaaquo ion, the vanadyl ion EPR[2] spectral line width can serve as a useful probe of intracellular pH (Frank et al., 1986). For *A. ceratodes* vanadocytes, this method yielded an intravanadophoric pH value of 1.8 ± 0.1.

Recently, we reported analytical evidence (Frank et al., 1986) that most of the soluble vanadophoric sulfur in *A. ceratodes* is represented by sulfate (1.3 M) and ~0.74 M is represented by non-sulfate sulfur. The oxidation state of the latter was indicated by XANES (X-ray absorption near-edge structure) spectroscopy to be slightly less than 6+ (Hedman et al., 1986). Here we present the results of detailed XANES investigations of an intact, living plasma cell preparation from *A. ceratodes*, yielding the first unambiguous evidence for large quantities of intracellular sulfate. The second form of sulfur, present in comparable concentration, is similarly found to be an aliphatic sulfonic acid, analogous to cysteic acid.

Experimental Procedures

Ascidia ceratodes were collected from the Monterey Yacht Harbor, Monterey, CA, maintained at 4 °C in aerated seawater, and used within 6 days. Half the water was exchanged with fresh 4 °C seawater every 24–48 h. The organisms were determined to be healthy by the presence of feces (which were removed periodically) in the holding tank, by response to stimulus, by a strong heartbeat, and by a firm, healthy looking integument. Plasma cells were taken from healthy individuals by heart puncture with sterile plastic syringes. The blood was immediately transferred into cold sterile plastic tubes embedded in ice, where the cells were allowed to slowly agglutinate into a cohesive mass. Such agglutination appears to be characteristic of blood from *Ascidia* as it has been observed to occur reversibly in vivo in agitated animals (Vallee, 1968; R. M. K. Carlson, unpublished observations; Wright, 1981) as well as in vitro in whole blood preparations (Vallee, 1968). This phenomenon can be inhibited or reversed by the addition of thiol reagents. Following collection, the cells were washed by 3 times repeated suspension/centrifugation (1000*g*, 5 min) in sulfate-free ascidian physiological saline (Carlson, 1976) (NaCl, 0.425 M; KCl, 0.00905 M; $CaCl_2$, 0.00860 M; $MgCl_2$, 0.0485 M; $NaHCO_3$, 0.092 M; pH adjusted to 6.6 with dilute HCl). The agglutinated cell mass was kept in ice until use (~1 h), and XANES spectroscopic data were collected at ambient temperature.

Ascidian physiological saline was prepared by modifying the Woods Hole Marine Biological Laboratory synthetic seawater formula (Cavanaugh, 1956) in accordance with ascidian plasma analyses as reviewed in Goodbody (1974). The pH and specific gravity of the saline were adjusted to values determined for *A. ceratodes* (pH 6.68 and sp. gravity = 1.022 for 25 mL of pooled plasma at 12 °C) (Carlson, 1976). No protein was added to this synthetic plasma in view of the finding of only 1.09 mg/mL protein (Carlson, 1976) for the native plasma from this organism, according to the Lowry method (Lowry et al., 1951). The above-mentioned sulfate-free plasma was prepared by substituting appropriate stoichiometries of metal chloride salts for corresponding metal sulfates.

Cysteine, methionine, methionine sulfoxide, methionine sulfone, cysteic acid, myoinositol hexasulfate, and 2-aminoethyl sulfate were purchased from Sigma Chemicals and used as received. All other chemicals were of reagent grade. Water was doubly deionized throughout. All solid samples were ground to fine powders and dispersed thinly on Mylar tape for the recording of the X-ray absorption data.

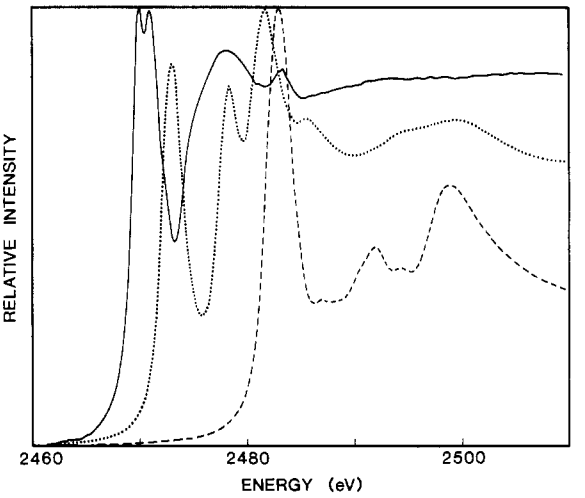

FIGURE 1: Sulfur *K*-edge X-ray absorption spectra measured in fluorescence mode for solid samples of $(NH_4)_2MoS_4$ (solid line), $Na_2S_4O_6 \cdot 2H_2O$ (dotted line), and Na_2SO_4 (dashed line). The S_4O_6 dianion contains two types of sulfur in different formal oxidation states. The terminal sulfurs are in an electronic environment similar to that in the SO_4^{2-} ion while the central sulfurs more nearly resemble their elemental form. This is clearly reflected in the edge features.

XANES spectral data of all biological samples and of the aqueous solutions were collected by fluorescence detection at the Stanford Synchrotron Radiation Laboratory (SSRL) on beam line VI-2 during dedicated conditions at 3 GeV and 25–60 mA, with the SSRL/Exxon/Lawrence Berkeley Laboratory 54-pole wiggler operated in undulator mode. The experimental setup and its advantages in collecting data for low-*Z* elements have been described (Hedman et al., 1986). Spectra of solid samples were measured with an identical experimental setup but under unfocused wiggler conditions on beam lines IV-2 and VII-3 at SSRL.

The data were measured from ~2420 to ~2740 eV with a step size of approximately 0.15 eV in the edge region (2450–2490 eV). Between two and four scans were collected for each sample. The energy was calibrated by use of X-ray absorption spectra of elemental sulfur, run at intervals between the samples. The often-called "white-line" maximum of the calibration spectra was assigned to 2472.7 eV, which corresponds to an inflection point of 2471.3 eV. The spectrometer resolution was of the order 0.5–0.6 eV. Repeated experiments have shown that the reproducibility in determining peak positions, by calculation of the first and second derivatives of the spectra, is about 0.2 eV. A smooth preedge background was removed from the spectra by fitting a polynomial to the preedge region and subtracting this polynomial from the entire spectrum. The presented spectra represent the average of the individual scans.

Results and Discussion

As illustrated in Figure 1, XANES spectra are characterized by a smooth preedge region, followed by the steeply rising absorption edge, upon which is superimposed features derived from transitions between the absorber 1s atomic orbital (for the *K* edge) and symmetry-available atomic or molecular orbitals. XANES thus contains information regarding electronic and geometric aspects of the absorber and its surrounding atom shell. Finally, the continuum region is reached

[2] Abbreviations: XANES, X-ray absorption near-edge structure; XAS, X-ray absorption spectroscopy; EXAFS, extended X-ray absorption fine structure; eV, electronvolt; EPR, electron paramagnetic resonance.

wherein resonance scattering of the virtually ionized photoelectron from nearest-neighbor and more distant atoms begins to contribute to the spectral features. At somewhat higher energies ($\simeq$50 eV above the edge) scattering effects predominate, and the extended X-ray absorption fine structure (EXAFS) spectral region begins.

Until recently, the notion of high intracellular sulfate concentrations within vanadocytes has been controversial (Bell et al., 1982; Dingley et al., 1982; Pirie & Bell, 1984). Following analytical experiments designed to address this problem (Frank et al., 1986) (see below), we undertook to measure the sulfur XANES spectra of whole cell preparations from the blood of *A. ceratodes* so as to render the analytical results unambiguous. Initially, data were gathered on simple sulfur-containing model compounds. Assigning the defining locus to be the first inflection point of the steeply rising absorption edge, a spread of nearly 13 eV was noted between sulfur of nominal oxidation state 2– (MoS_4^{2-}) and sulfur of 6+ (SO_4^{2-}) (Figure 1). Near-edge spectra of a variety of sulfur compounds were each found to be richly endowed with characteristic features, allowing ready qualitative discrimination among various oxidation states and structures. For example, the organosulfate esters 2-aminoethyl sulfate and myoinositol hexasulfate have superimposable spectra and, though at the same formal 6+ oxidation state as sulfate, display a second sharp edge feature 1.8 eV to lower energy than the main peak maximum at 2482.4 eV (sulfate exhibits a single peak at 2482.5 eV). We are presently engaged in self-consistent field X–α calculations to bring a detailed quantitative understanding to the origin of the observed spectral features.

Figure 2a shows the sulfur XANES spectrum arising from a plasma cell preparation from *A. ceratodes* prewashed with sulfate-free synthetic plasma. On first inspection, clear similarity to a sulfate spectrum is noted in the intense white-line absorption maximum at 2482.4 eV. The very good signal to noise ratio clearly confirms as endogenous the high concentration of sulfate previously found on atomic emission analysis (Frank et al., 1986), spectroscopic analysis (Bell et al., 1982), and gravimetric analysis (Carlson, 1975; Frank et al., 1986) of plasma cell lysates as well as the sulfur detected by X-ray microanalysis of fixed cell sections (Bell et al., 1982; Rowley, 1982; Pirie & Bell, 1984). The smaller feature at 2473.7 eV can be assigned to a more highly reduced sulfur type probably reflecting cellular thiol (cysteine) (2473.5 eV) and/or thioether (methionine) (2473.7 eV) components. Indeed, the major obvious features of the cellular spectrum are duplicated in Figure 2b, showing the spectrum obtained from a solution containing 10 mM cysteine and 100 mM sodium sulfate.

However, the whole cell spectrum also shows a lower energy shoulder, attendant to the main peak, that was obviously manifest as an extra inflection at 2481.7 eV in the first- and second-derivative spectra (not shown). In our previous analytical experiments, 63% of the endogenous soluble sulfur, derived from plasma cell lysates, precipitated with Ba^{2+} and was identified thereby as sulfate. This assignment was confirmed by X-ray fluorescence analysis of the $BaSO_4$ precipitate. The remaining 37% of the sulfur proved to be in an alternative form by its inability to be precipitated by Ba^{2+} ion. This unknown sulfur type calculated to an average cellular concentration of 54 mM or a vanadophoric concentration of 0.74 M. For sulfate, these values were 92 mM and 1.3 M, respectively.

The spectrum of sulfate within a solution of 1 M vanadium(III) sulfate, pH 1.5, is shown in Figure 2c. This milieu more closely approximates the conditions now considered to

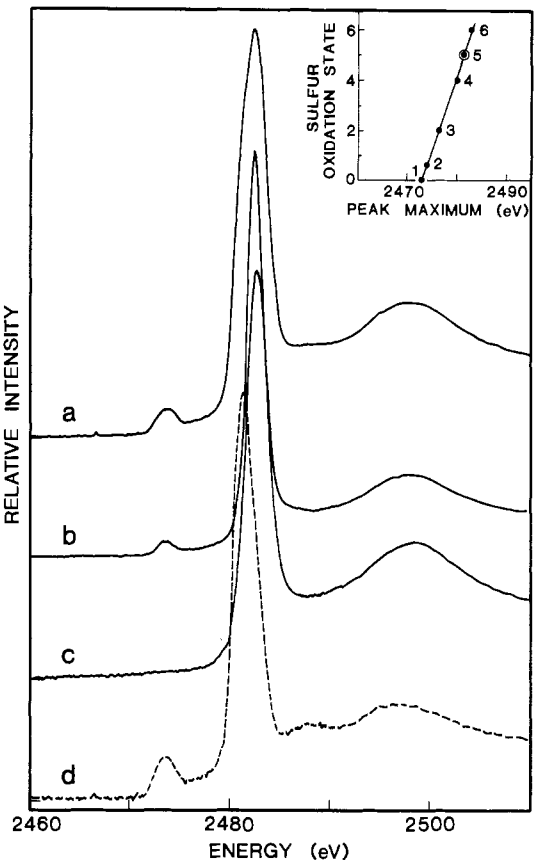

FIGURE 2: Sulfur *K*-edge X-ray absorption spectra of (a) a plasma–cell pack washed with sulfate-free synthetic plasma, (b) an aqueous solution of 100 mM Na_2SO_4 and 10 mM cysteine, and (c) vanadium(III) sulfate, 1 M in pH 1.5 H_2SO_4 [note the increased white-line width relative to the sulfate line in (b)]. (d) Weighted (see text) difference spectrum, (a) minus (c). (Inset) Plot of sulfur *K*-edge X-ray absorption white-line maxima (in eV) vs. oxidation state for (closed points) (1) elemental sulfur, (2) methionine (cysteine), (3) methionine sulfoxide, (4) methionine sulfone, (5) cysteic acid, and (6) sulfate. The data were fitted with a linear least-squares line (r = 0.999). Assignment of zero oxidation state to elemental sulfur imposed an apparent valence state of 0.6+ for sulfur in cysteine and methionine. The remaining points were assigned according to the formal oxidation state of sulfur, as determined by each oxo group having a 2– charge. Thus, methionine sulfoxide sulfur is 2+. These assessments are reasonable in view of the various group and atom electronegativities (Hinze & Jaffe, 1962, 1963; Hinze et al., 1963) and electron densities (Van Wazer & Absar, 1975). The open point around 5 is the datum obtained from difference spectrum d, above, which overlays that for cysteic acid exactly.

prevail within vanadophores from *A. ceratodes* (Frank et al., 1986). It is noteworthy that the width at half-height of the white-line absorption feature (2.9 eV) is 33% wider than that of sodium sulfate, 1 M in pH 1.5 sulfuric acid solution (2.2 eV). Interaction between V(III) and sulfate may be at least in part responsible for this effect. Thus, the sulfur within a VSO_4^+ complex ion (Britton & Welford, 1940; Kimura et al., 1972; Bielig et al., 1966; Duffy & Macdonald, 1970; Carlson, 1975; Frank et al., 1986) may contribute an absorption peak slightly removed from that of the fully solvated counterion. Possible mechanisms include perturbation of the energy of sulfate valence orbitals or the lifting of the degeneracy from the T_2 sulfate valence state by complexed vanadium(III). A third source of broadening may involve concentration-dependent variation in line shape, arising from the fact that the data represent excitation fluorescence spectra (Melhuish, 1982). Thus, we have recently noted self-absorption (inner filter) effects at absorber concentrations $\geq$100 mM, as seen in the difference in relative heights of white-line and continuum features for solutions of varying concentrations having oth-

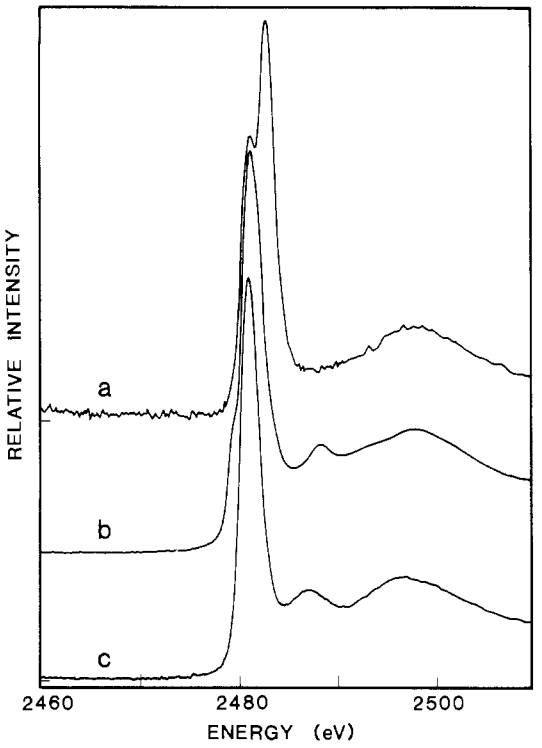

FIGURE 3: Sulfur *K*-edge X-ray absorption spectra of (a) myoinositol hexasulfate, 25 mM in dilute aqueous NaOH, (b) *p*-toluenesulfonic acid, 1 M in water, and (c) cysteic acid, 0.5 M in water.

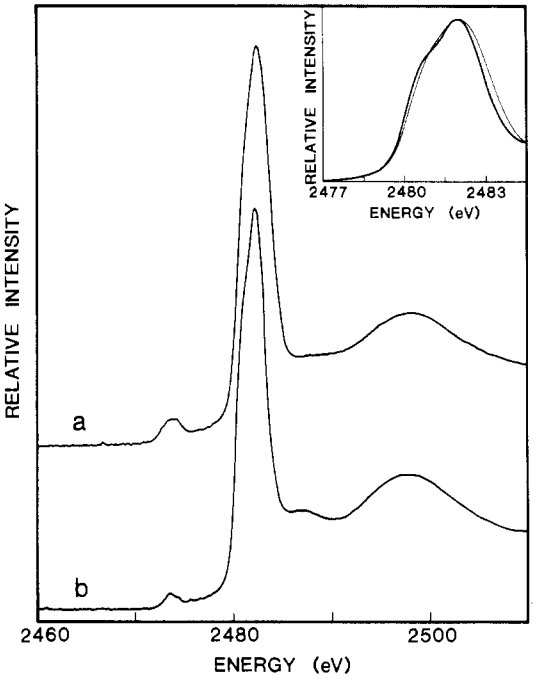

FIGURE 4: Sulfur *K*-edge X-ray absorption spectra of (a) washed cells (see text, Figure 2a) and (b) a solution of cysteine (50 mM), cysteic acid (0.5 M), and sodium sulfate (0.5 M) in 2 M ammonium acetate pH 4. Note the relative line-broadening effect in (a) which characterizes vanadium(III) sulfate solutions and apparently characterizes sulfate and sulfonate in vanadocytes. (Inset) Edge region of spectrum a (light) and spectrum b (dark), expanded and overlaid.

erwise identical (or very similar) sulfur types (cf. Figure 2b,c). Discrimination among these possibilities is presently under active investigation.

In order to obtain a spectrum of the sulfur type responsible for the 2481.7-eV shoulder in the washed-cell spectrum, a series of difference spectra (2a minus 2c above) was generated following the scaling of the normalized vanadium(III) sulfate spectrum to reflect various proportions of sulfate and centered about the fraction analyzed to be present as soluble sulfate. The best resultant difference spectrum was judged by noting the appearance and growth of a dip at 2485.1 eV, which became noticeable at a washed-cell sulfur to sulfate ratio of 1.0:0.56 and the prominence of which increased as the fraction of sulfate subtracted became larger. The spectrum chosen by this criterion is shown in Figure 2d and reflects a proportionality of washed-cell sulfur to sulfate of 1.0:0.52. The residual intensity difference spectrum shows features again somewhat reminiscent of sulfate, but with a white-line maximum 1.2 eV to lower energy and containing an extra feature at 2488.2 eV, which was present throughout the above-mentioned series.

In the inset of Figure 2 is shown plotted the position of the peak maximum (in electronvolts) vs. oxidation state for a series of related sulfur-containing compounds (cf. figure legend). All are characterized by a single sharp white-line maximum above the absorption edge. The data were scaled to assign a valence of zero to elemental sulfur and a hexavalent state to sulfur within sulfate. The energy position of the difference maximum from Figure 2d overlays exactly the point for cysteic acid, indicating an apparent oxidation state of 5+ for sulfur in the unknown compound.

In Figure 3 are shown the aqueous solution spectra of cysteic acid, *p*-toluenesulfonic acid (tosic acid), and myoinositol hexasulfate ester. Clear differences in edge shape between the sulfonic acids are seen in the shoulder at 2479.6 eV in the tosic acid spectrum, probably accruing to the aromatic moiety, and in the shape of the respective continuum features near 2488 eV. Both sulfonic acid spectra are readily distinguished from that of the sulfate ester, the main absorption maximum of which is strongly split. Comparison of the number and positions of the various absorption features of these with the difference spectrum in Figure 2d is essentially sufficient to establish the identity of the latter with the spectrum of cysteic acid. The difference spectral features are somewhat broadened relative to those of cysteic acid however (e.g., white-line half-height widths are 3.0 and 2.6 eV, respectively).

In Figure 4, comparison is made between the washed-cell spectrum (Figure 2a) and that of a model solution consisting of cysteine, cysteic acid, and sodium sulfate in a ratio of 0.1:1.0:1.0. Detailed comparison of the white-line region is made in the inset. Since the model solution contains no vanadium(III), it is not expected to reproduce the broadening observed in the plasma cell spectrum. Experiments are currently in progress to model this spectrum with solutions that reproduce more adequately the intravanadophoric milieu experienced by sulfur. The model solution spectrum does, however, provide evidence for the validity of the assessment that both sulfate and sulfonate contribute to the line shape of the cellular spectrum. Thus with the exception of cysteine concentration and the broadening effects previously mentioned, the inorganic solution spectrum is seen to effectively reproduce the shape and relative intensities of the washed-cell spectral components. The unknown absorbing sulfur type in *A. ceratodes* plasma cells is thereby seen to be an aliphatic sulfonic acid with a total concentration close to that of sulfate.

The finding in *A. ceratodes* of an aliphatic sulfonic acid in so large a concentration is to our knowledge unprecedented among marine organisms. Various amino sulfonic acids of pelagic origin have been previously detected however, notably in algae (Fattorusso & Piattelli, 1980; Balzer, 1981) and diatoms (Busby & Benson, 1973; Anderson et al., 1975). We note that the possible (if presently speculative) derivative relationship of the sulfate/sulfonic acid reservoir with the

acidic mucopolysaccharides (Goldberg et al., 1951; Kalk, 1963; Krishnan, 1975; Flood & Fiala-Medioni, 1981) implicated in vanadium uptake and absorption by ascidians may have bearing on the mechanisms of transport and endocytosis of this metal. A potential solution is thereby also become available to the problem of acquisition of sulfate by vanadocytes, since uptake of this ion as such apparently does not occur (Dingley et al., 1981).

Finally, these studies demonstrate the utility of XANES spectroscopy in discovering novel materials in situ, especially those embedded in complex and amorphous biological milieux, and characterizing them nondestructively (Galloway, 1985).

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