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Vanadyl Ion EPR as a Noninvasive Probe of pH in Intact Vanadocytes from *Ascidia ceratodes*

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Analytical and EPR spectral measurements have been carried out to quantify the nature of sulfate, vanadium, and acidity in the vanadocytes of *Ascidia ceratodes*. Atomic emission spectroscopic data show that average per cell concentrations of vanadium and sulfur are 0.099 and 0.25 M, respectively. In the cell lysate from 0.85 mL of packed cells, 123.91 μmol of sulfur was found of which only 78.41 μmol precipitated with $BaCl_2$. X-ray fluorescence examination of the $BaSO_4$ precipitate showed no evidence of a vanadate contaminant. Extrapolation of vanadium and sulfate quantities to vanadophores yields endogenous concentrations of 1.4 and 1.3 M, respectively. Analytical data for Fe, Zn, Cr, Cu, and P are also reported. EPR spectroscopic examination of whole-cell preparations showed the presence of vanadyl ion, which was quantitated by spectral integration to 13 and 36 mM, respectively, within the vanadophoric cell constituents from two independent collections of animals. The magnetism exhibited by vanadyl ion within healthy, intact vanadocytes in room-temperature EPR spectra ($A_0 = 1.074 \times 10^{-2}$ cm^{-1}, $g_0 = 1.968$) and frozen-solution spectra ($A_\parallel = 1.814 \times 10^{-2}$ cm^{-1}, $g_\parallel = 1.935$) is shown by comparison with standards to be consistent only with the presence of the free aquo ion. Sources of EPR spectral broadening observed in the vanadocyte frozen-solution spectra ($(-7/2)_\parallel$ line width = 34.0 and 24.3 G in the two collections of animals relative to 12.5 G from 1 mM $VOSO_4$ in 100 mM H_2SO_4) were investigated by studying the effect on vanadyl EPR line width of acidity (as H_2SO_4), viscosity, SO_4^{2-} concentration, and high concentrations of the paramagnetic ions VO^{2+}, Co^{2+}, and V^{3+}. Broadening is shown to derive from at least two dimeric vanadyl species, the formation of one of which is enhanced by sulfate but inhibited by chloride or perchlorate. In solutions containing sulfuric acid, the aquovanadyl ion EPR line width is shown to be closely correlated with $[H^+]$ over the range 500 mM $\geq [H_2SO_4] \geq$ 1 mM. In the presence of 0.5 M $V_2(SO_4)_3$ in 15 mM H_2SO_4, the 31-G VO^{2+} EPR line width was found to accurately reflect the measured pH of 1.9. This line width/pH correlation, exploited to ascertain the internal vanadophoric pH in *A. ceratodes* vanadocytes, yielded a pH of 1.8 ± 0.1. This pH is sufficiently low to prevent hydrolysis of endogenous $V(H_2O)_6^{3+}$ or its autoxidation ($E_{1/2}(V^{3+}/VO^{2+}) = 283$ mV at pH 1.8). The most likely status for V(III) in vanadocytes at low pH and high $[SO_4^{2-}]$, keeping in mind previous EXAFS and ^{1}H NMR results, is as the $V(SO_4)(H_2O)_{4-5}^+$ complex ion. Finally, a new hypothesis for the utility of V(III) to tunicates is advanced.

Introduction

One of the more intriguing problems in inorganic biochemistry concerns the absorption, utilization, and status of vanadium in tunicates, (a group of mostly sessile, strictly marine, filter-feeding animals classified among the Chordata[1]). The remarkable ability of the varieties of this animal to selectively concentrate one or another transition metal from the oceanic reservoir, often by a factor of 10^6 or more, and to maintain it in an air-sensitive valence state, recommends this unprepossessing organism to study.[2-10]

In tunicates of the class Ascidiacea, vanadium is the principle metal concentrated in specialized plasma cells although much smaller quantities of iron[4,5] and copper[5] are also found. These greenish-hued cells are termed "vanadocytes", and the vanadium is concentrated within vacuolar processes called "vanadophores". The sizes of these structures are of the order of 10 and 2 μm respectively, and in excess of 14 vanadophores can be found per vanadocyte.

In general, the vanadophoric contents are thought to be markedly acidic,[7,8,11-13] although this has been disputed.[3,14] The vanadium is usually maintained in the air-sensitive trivalent (V(III)) state.[11,15] Aerobic lysis of the cells produces a deep red-brown mixture, termed the Henze solution.[11,16,17] Vanadophoric vanadium has been shown to have no direct role in plasma oxygen transport,[18] but has been implicated for use in discouraging predation[4,19] or surface fouling[20] by competing organisms. Other functions have also been suggested,[1,3,21] including involvement in tunic formation and immune response.

EPR spectroscopic examination[22,23] of the whole blood of *Ascidia nigra* revealed a small concentration of vanadyl ion to be present, with most of the vanadium being V(III). Vanadyl ion has also been detected by this method in vanadocytes from both *Ascidia mentula* and *Ascidiella aspersa*.[13] Vanadyl ion has heretofore, however, not been reported in vanadocytes from *Ascidia ceratodes*.[4]

^{1}H NMR measurements on living packed blood cells from *A. ceratodes*[24] indicated that the endogenous [V(III)] was maintained at 1.08 *m* (≃1.28 M) as the inorganic sulfate; four to five coordination sites being available for proton (and thus most likely water) exchange. These results were confirmed and extended by X-ray absorption spectroscopic (XAS) examination of living *A. ceratodes* vanadocytes,[25] which revealed the first coordination

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Table I. Major Metal and Sulfur Concentrations in *A. ceratodes* Whole Blood[a]

	S	V	Fe	Zn	Cr	Cu
cell debris	82.50	39.79	0.019	0.031	*b*	0.02
pellet	3.11	1.12	0.020	0.016	3.7×10^{-3}	7×10^{-4}
lysate	123.91	43.71	0.350	1.60	*b*	*b*
total (cell)	209.52	84.53	0.389	1.65	3.7×10^{-3}	0.021
plasma[c]	20.2	0.144	0.006	0.10	1.8×10^{-3}	*b*
seawater[c,d]	27.6	3.9×10^{-5}	1.8×10^{-4}	1.5×10^{-4}	9.6×10^{-7}	4.7×10^{-5}

[a] Quantities are given in μmol of the element found/0.85 mL of packed cells. Thus average per cell concentrations are, e.g., 0.099 M for V and 0.25 M for S, assuming no interstitial water in the cell pack. Of the total lysate sulfur, only 78.41 μmol could be precipitated with $BaCl_2$. [b] Not detected. [c] Micromoles per milliliter. [d] Data taken from ref 32.

Table II. EPR Spectral Data for Vanadocytes and Aquovanadyl Ion

	line width[a]	pH	$A_\parallel$[b]	$g_\parallel$	A_0[b]	g_0	$[VO^{2+}]^f$
			Frozen Sample				
packed cells[c]	24.3, 34.0	1.7, 1.9	1.814	1.935	1.091	1.973	13, 36 (0.91, 2.6)
single animal no. 2	33.5	1.9	1.82	1.94	1.08	1.974	
single animal no. 3	31.4	1.9	1.83	1.94	1.09	1.978	
Henze soln	17.3, 18.4		1.825	1.934	1.081	1.971	5.5, 8.7
$VO(H_2O)_5^{2+}$[d]	12.5		1.833	1.935	1.090	1.974	1
$VO^{2+}/V(III)$[e]	34.0	1.9	1.822	1.937	1.083	1.974	12
			Liquid Sample				
cell suspension	38				1.074	1.968	
$VO(H_2O)_5^{2+}$[d]	30				1.068	1.968	1
$VO^{2+}/V(III)$[e]	40				1.060	1.967	12

[a] Width at half-height of the $(-7/2)_\parallel$ EPR line (frozen solution); +7/2 line (liquid solution). [b] In units of $cm^{-1} \times 10^2$; structurally significant variation in A is $\pm 0.02 \times 10^2$ cm^{-1}.[29,33] The isotropic coupling constants were calculated from $A_0 = (A_\parallel + 2A_\perp)/3$ in cm^{-1}. [c] Two independently collected samples, 5 years apart. The integrated $[VO^{2+}]$ must be multiplied by the factor 13.8 to correct for the ratio of vanadophore volume to packed cell volume.[24] The parenthetical values are $[VO^{2+}]$ averaged over the whole sample volume and are, therefore, more directly comparable to the Henze solution concentrations. [d] In 100 mM H_2SO_4. [e] VO^{2+} ion, 12 mM with 0.5 M $V_2(SO_4)_3$ in 15 mM H_2SO_4. The g and A values were essentially invariant over 1 mM $\leq [VO^{2+}] \leq$ 20 mM. [f] In units of mM.

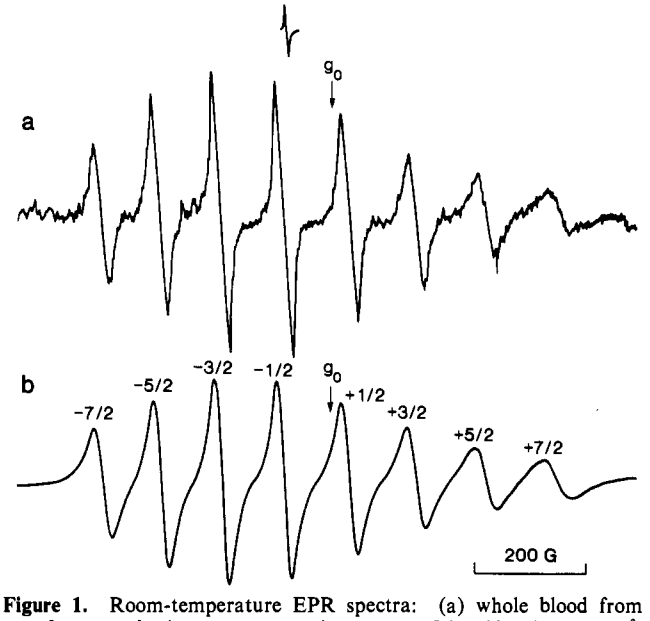

Figure 1. Room-temperature EPR spectra: (a) whole blood from *Ascidia ceratodes* (spectrometer settings: power 5.0 mW, gain 4.0×10^3, modulation amplitude 8.0 G, frequency 9.525 GHz); (b) 12 mM VO^{2+} with 0.5 M $V_2(SO_4)_3$ in 15 mM H_2SO_4. (Spectrometer settings were power 10.0 mW, gain 2×10^3, modulation amplitude 8.0 G, and frequency 9.535 GHz). The pitch signal marks 3392.8 G.

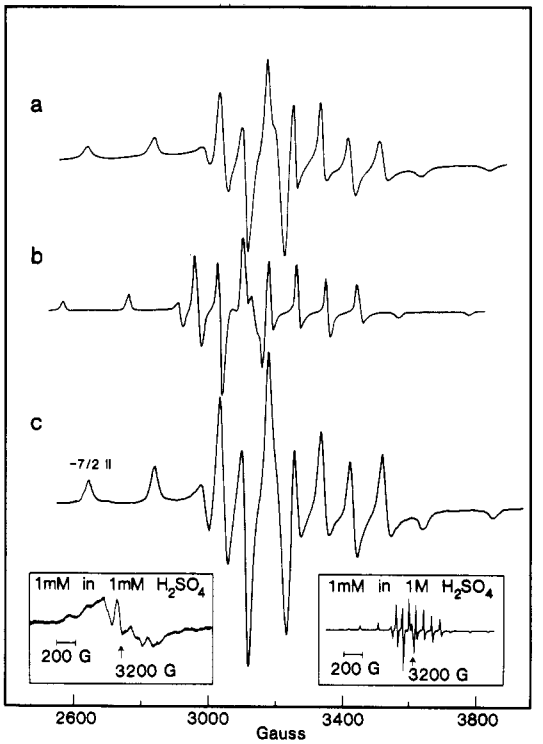

Figure 2. Frozen-solution EPR spectra of vanadyl ion: (a) in vanadocytes, packed column (2000*g*, 10 min); (b) in the Henze solution, prepared by thawing the sample used in part a, heating to 95 °C for 30 s, and then freeze-quenching in liquid N_2; (c) in a 1 mM solution in 11 mM H_2SO_4. Insets: left, 1 mM solution in 1 mM H_2SO_4; right, 1 mM solution in 1 M H_2SO_4. Conditions: temperature near 110 K; (a, b) spectrometer power 12.5 mW, modulation amplitude 4.0 G; (c) power 10 mW, gain 2.5×10^4, modulation 5.0 G; (left inset) as in part c but with gain 1.25×10^5; (right inset) power 4 mW, gain 1.25×10^4, modulation 4.0 G.

sphere of vanadium(III) to consist of six oxygens at an average distance of 1.99 Å. No evidence for a ligand second shell was found, rendering unlikely the possibility that the vanadium is confined either by a rigid low MW chelate or a highly ordered protein binding site. It was further found that the Henze solution also contained vanadium principally in the trivalent state, with an upper limit of 10% placed upon vanadyl ion concentration, this most likely arising from air oxidation.

In the course of these XAS studies it was found, in contrast to the previous report,[4] that fresh samples of packed blood cells

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Table III. EPR Line Width Dependence of Vanadyl Ion with Added Solutes in Frozen Solution

$[VOSO_4]$, mM	fwhh, G	solute (concn, mM)	$[H_2SO_4]$, mM
1	v br	$CoSO_4$ (500)	1
1	v br	$CoSO_4$ (500)	5
1	*a*	$CoSO_4$ (500)	10
1	*a*	$CoSO_4$ (500)	50
1	~31[b]	$CoSO_4$ (500)	100
1	~26[b]	$CoSO_4$ (500)	200
1	~17[b]	$CoSO_4$ (500)	500
10	17.3	$CoCl_2$ (500)	200
1	30	$V_2(SO_4)_3$ (500)	15
11	33	$V_2(SO_4)_3$ (500)	15
1	17	NaCl (50)	1
1	22	$NaClO_4$ (50)	1

[a] Convolution of sharp and broad spectral components (see text). [b] As in footnote *a*, but with the sharp component dominant.

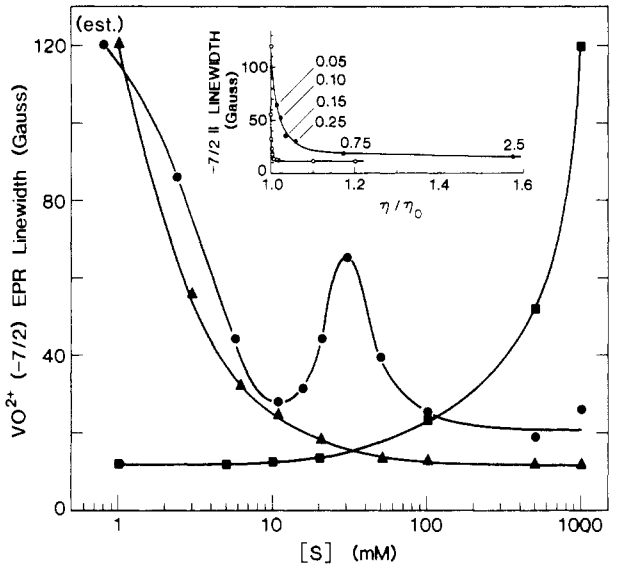

Figure 3. EPR $(-7/2)_{\parallel}$ line width dependence in frozen solution of vanadyl ion: (▲) for a 1 mM solution as a function of added H_2SO_4; (■) as a function of concentration in 1 M H_2SO_4; (●) for a 1 mM solution in 1 mM H_2SO_4 as a function of added Na_2SO_4. [S] = concentration of added solute. Inset: Line width displayed as a function of relative viscosity. The additives are poly(vinylpyrrolidone) for closed points (Included numbers refer to solution composition (% w/w); viscosity was calculated from the equation given in ref 39.) Sulfuric acid for open points. H_2SO_4 concentrations (mM): 1, 5, 10, 20, 30, 40, 50, 100, 500, 1000. Viscosity was taken or interpolated from the data given in ref 66.

from five independent collections of *A. ceratodes* exhibited an EPR signal indicative of vanadyl ion, both at ambient and liquid-nitrogen temperatures.[26] The concentration of this ion was estimated as ≲1% of the total vanadium by comparison with the EPR spectra of standard vanadyl ion solutions. In view of these findings, a more thorough study of this phenomenon was undertaken, since vanadyl ion may participate in the functional role of vanadocytes. In addition, it seemed possible that intrinsic vanadyl ion could serve as a completely noninvasive probe of conditions within these cells.

We report here an EPR study of vanadium in both frozen and liquid whole-cell preparations from healthy specimens of *A. ceratodes*. The effects of acidity (as H_2SO_4), added sulfate ion, viscosity, and paramagnetic ion additives, including vanadyl ion itself, on the EPR line widths of frozen inorganic vanadyl ion solutions were also investigated. In addition, analytical data relating to cellular sulfur and vanadium as well as a variety of other metals have been acquired. The results are applied to an understanding of the distribution of sulfur and the status of va-

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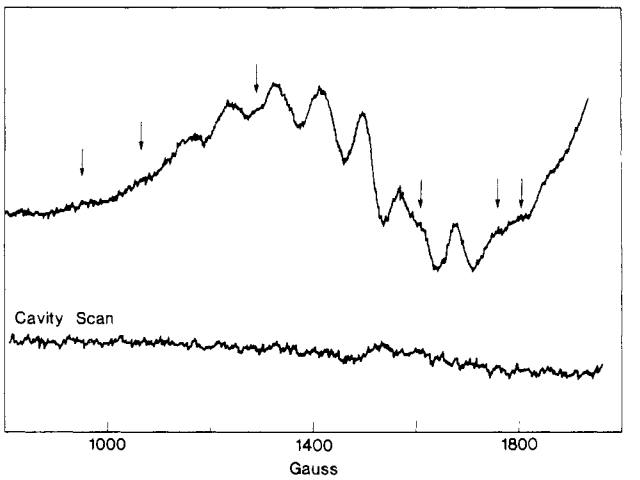

Figure 4. Frozen-solution $g = 4$ vanadyl dimer EPR spectrum. The vanadyl ion concentration was 0.5 M with 0.5 M H_2SO_4 in 50% v/v glycerol. The solution froze to a clear glass. Similar spectra were obtained from 0.5 M $VOSO_4$ in 1 M H_2SO_4 only. Seven lines are clearly visible, and arrows mark points of obvious additional structural features. A portion of the high-field side is obscured by residual intensity tailing from the $g = 2$ portion of the spectrum. Spectrometer settings were power 25 mW, gain 1.25×10^5, modulation amplitude 8.0 G, frequency 9.04 GHz.

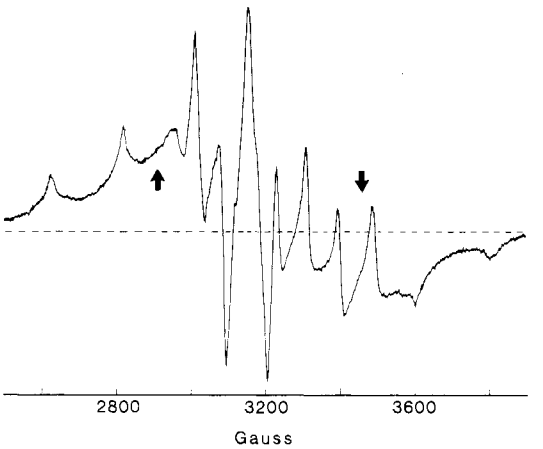

Figure 5. EPR spectrum of 1 mM $VOSO_4$ with 30 mM Na_2SO_4 in 1 mM H_2SO_4 showing evidence for the superposition of broad and narrow spectral elements (arrows). Compare this figure with Figure 2c. The dashed line marks the baseline.

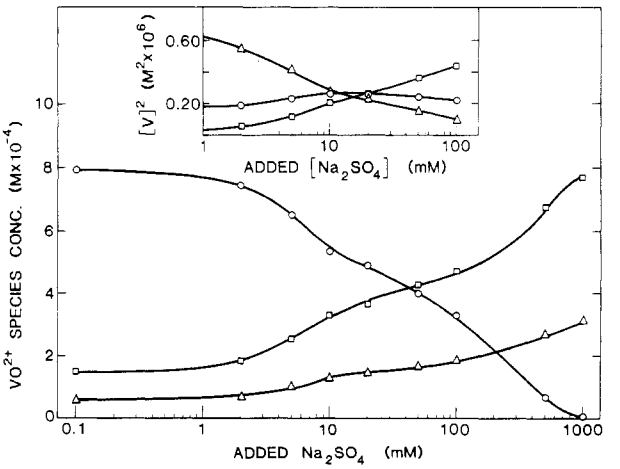

Figure 6. Calculated concentration dependence of vanadyl ion complexes as a function of added sodium sulfate (total $[VO^{2+}] = 1$ mM): (○) $[VO(H_2O)_5^{2+}]$; (△) $[VO(H_2O)_5SO_4]$; (□) $[VO(H_2O)_4SO_4]$. Inset, square law dependence as a function of added sulfate: (△) $[V]^2 = [VO(H_2O)_5^{2+}]^2$; (○) $[V]^2 = ([VO(H_2O)_5^{2+}])([VO(H_2O)_5SO_4] + [VO(H_2O)_4SO_4])$; (□) $[V^2] = (([VO(H_2O)_5SO_4] + [VO(H_2O)_4SO_4])^2)$.

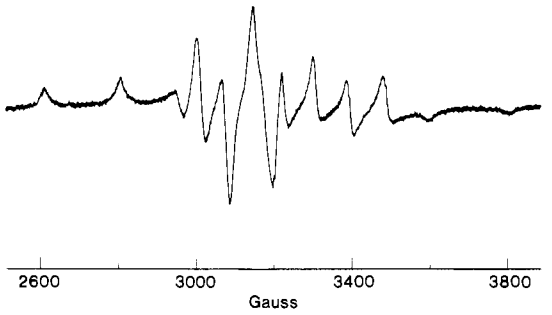

Figure 7. EPR spectrum of 2 mM $VOSO_4$ with 0.5 M $V_2(SO_4)_3$ in 15 mM H_2SO_4, frozen solution. Spectrometer power 4 mW, gain 6.2×10^4, modulation 6.3 G, $T \sim 110$ K, frequency 9.07 GHz.

nadium in vanadocytes. The question of intravanadophoric acidity is addressed by the development of vanadyl ion frozen solution EPR line width as a noninvasive probe of pH.

Nomenclature

EPR: electron paramagnetic resonance
XAS: X-ray absorption spectroscopy
EXAFS: extended X-ray absorption fine structure
ICP: inductively coupled plasma

Materials and Methods

Vanadocyte Preparations. *A. ceratodes* specimens were collected at the Monterey Yacht Harbor in Monterey, CA. The specimens were kept in cool fresh sea water before and during transport to Stanford (~2 h) and thereafter were kept at 4 °C in aerated seawater.

Within 24 h of collection, the blood from ~100 healthy animals was taken by heart puncture, using sterile plastic syringes (Stylex tuberculin). An average of ca. 0.5 mL of blood was obtained per animal, and this was pooled at 0 °C and used within 24 h. For frozen-cell EPR spectroscopy, the green agglutinated cell mass was gently transferred by means of Pasteur pipets directly into ice-cold 4-mm quartz EPR tubes, which had been prewet with plasma. Care was taken to avoid contact between pipet and EPR tube wall so as to forestall inadvertant lysis of these rather fragile cells. The cell mass was then packed in the tubes at 4 °C by centrifugation (ca. 2000*g*). The blood from approximately 30 animals was sufficient to obtain a 2.5-cm-high column of packed cells. Before EPR analysis, the packed cells were visually inspected for evidence of lysis as indicated by blue or brown specks. No specks were noted even after storage in the packed state at 0 °C for 1–2 days. For single-animal spectra, the agglutinated cell mass was resuspended into a 2.5-cm column of plasma within the EPR tube. The suspension was then rapidly frozen in liquid nitrogen.

The Henze solution was prepared as described in the legend to Figure 2.

Vanadium and Sulfur Analysis. To verify and quantitate the presence of sulfate in vanadocytes, 0.85 mL of packed whole blood cells (3000*g*, 15 min) was lysed in 5 mL of anaerobic HCl solution (pH 2), followed by centrifugation (3000*g*, 15 min). The cell debris were twice washed by resuspension/centrifugation in the above HCl solution. The clear brownish supernates were combined and diluted to 10.25 mL with use of the same pH 2 HCl. The diluted lysate was then centrifuged overnight (3000*g*) under an argon blanket to remove a reddish microparticulate sediment. The obtained pellet was retained for further analysis (vide infra).

From the 10.25 mL of lysate, 2.0 mL was removed for separate ICP analysis described below. The remaining 8.25 mL was treated with 2.0 mL of 1 M $BaCl_2$. The white precipitate was collected by centrifugation and washed by suspension/centrifugation until judged free of chloride by the lack of a precipitate on addition of $AgNO_3$ solution to the supernate. The initial supernate solution was proved free of sulfate by addition of further $BaCl_2$. Following lyophilization and finally heating under vacuum, 14.7 mg of $BaSO_4$ was collected. This material was checked for contamination by vanadate by using X-ray fluorescence spectroscopy.

The *cell debris*, the *pellet*, the *lysate* (2.0 mL; see above) and the cell-free *plasma* were each analyzed for vanadium, sulfur, and a variety of other elements. The former three fractions were digested in concentrated HNO_3 and taken to dryness. The solutions resulting from dissolution of the residues were then subjected to ICP atomic emission spectroscopic analysis. The plasma sample was diluted 1:10 and analyzed directly. Samples were run in duplicate and concentrations determined by comparison with standards.

Vanadium(III) Solution Preparations. Pure vanadium foil (0.2754 g; Alfa Ventron 99.7% V) was dissolved anaerobically at 100 °C in 2.35 g of H_2SO_4 (Alfa Ventron ultrapure 96+%) in 8 mL of deoxygenated doubly deionized water. The resulting dark green solution was diluted anaerobically to 13.25 mL. This solution was found to be 8.6 mM in vanadyl ion by integration (peak height × width at half-height) of the $(-7/2)_{\parallel}$ EPR line[27-29] and comparison with standards; thus [V(III)] was 0.399 M. A second V(III) solution containing 21.7 mM vanadyl ion was prepared directly by dissolving $VOSO_4.H_2O$ (K&K Laboratories) in the previous solution.

Solutions of 1.0 M V(III) in 15 mM H_2SO_4 were prepared by first dissolving $VOSO_4$ to 1.0 M in 0.515 M H_2SO_4. Following addition of 100 mg of 10% Pd/C, H_2 gas, prehumidified through 0.515 M H_2SO_4, was bubbled through the VO^{2+} solution. After 70 h the Pd/C was removed by centrifugation under an argon blanket (9000*g*, 10 min). The deep green solution was found to contain 1.2 mM residual vanadyl ion. One molar vanadium(III) solutions containing higher concentrations of vanadyl ion were prepared by adding the requisite quantity of anaerobic 0.1 M $VOSO_4$ in 15 mM H_2SO_4 to 1.0 mL of the V(III) solution. The pH of these, as well as the above 1.0 M V(III) in 15 mM H_2SO_4 solution, was 1.9 (~22 °C).

All anaerobic work was carried out either under an argon atmosphere (99.98% Ar) in a glovebag or inside a Vacuum Atmospheres glovebox under dinitrogen purified through a BTS catalyst purge. Vanadium metal was dissolved under prepurified argon (Matheson Gas) with use of Schlenk apparatus.

Frozen-solution EPR spectra were recorded near liquid-nitrogen temperature (ca. 110 K) on either a Bruker ER 220D-SRC spectrometer or a Varian E-3 spectrometer, both operating in the X-band. Room-temperature X-band spectra were acquired on a Varian E-112 EPR spectrometer. EPR line width determinations in frozen solution spectra were made by measuring the width at half-height of the low-field ($(-7/2)_{\parallel}$) line. The EPR spectral sweep width was reduced to ±100 or ±250 G at these times.

For the viscosity experiments, Sigma pharmaceutical grade poly(vinylpyrrolidone) (mol wt 40000) was used. Vanadium, sulfur, and other elements were quantitated by plasma emission spectroscopy using a Bausch and Lomb-Applied Research Laboratories Model 3580 inductively coupled plasma Quantovac atomic emission analytical system. The $BaSO_4$ sample arising from the lysate sulfate analysis was checked for vanadium by means of X-ray fluorescence spectroscopy using a Kevex 0700/7000 spectrometer with a rhodium anode X-ray tube and an iron secondary target at 15.0 kV and 1.0 mA.

Unless otherwise specified, reagents were of analytical quality and used as received. All water was doubly deionized.

Results and Discussion

1. Vanadium and Sulfate in Blood Cells. Because the notion of high sulfate concentration within vanadocytes has become controversial,[12-14] it was decided to assess the presence of this counterion in packed blood cell samples from *A. ceratodes*. Although appreciable sulfur has been localized in vanadocytes by electron microprobe,[12,13] its identity as sulfate cannot thus be ascertained. The results from gravimetric analysis[24] have been called into question[14] because of the possibility of coprecipitation by Ba^{2+} of vanadate ion. This species could conceivably arise from oxidation of released vanadic ion by dissolved dioxygen following aerobic lysis.

In the analytical procedure reported here, autoxidation of V(III) was avoided by anaerobic acidic cell lysis. However, as a further check, the $BaSO_4$ gravimetric sample was analyzed for the presence of vanadium by X-ray fluorescence spectroscopy. This method has a vanadium detection limit of several parts per million. Vanadium concentration in the $BaSO_4$ precipitate was below the detection limit, whereas intense barium and sulfur peaks were observed.

In all, 18.3 mg of $BaSO_4$ were obtained from 0.85 cm^3 of packed cells. Assuming no interstitial water in the cell pack, this yields an average cellular concentration of 0.092 M sulfate. From the microscopic/^1H NMR data in ref 24, assuming all the sulfate is within the vanadophore, a concentration of 1.3 M can be calculated for the level of sulfate within this organelle. As discussed below, this value is consistent with extrapolated vanadium concentrations.

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Total cellular sulfur was analyzed by means of ICP atomic emission spectroscopy along with total vanadium, iron, zinc, chromium, and copper. The results are given in Table I. The observed quantity of sulfur and its distribution (vide infra) are remarkable. In the total lysate, 123.91 μmol of sulfur was found. Of this, 78.41 μmol is due to sulfate. The remainder, 45.50 μmol, or fully 36.7% of the total lysate sulfur, was present in a form that is not precipitated by Ba^{2+}. If within the vanadophore, the concentration of this latter sulfur form is 0.74 M. These findings have been recently strengthened by means of sulfur K-edge X-ray absorption studies using synchrotron radiation. XAS examination of washed whole-cell preparations from the blood of *A. ceratodes* revealed an intense absorption edge indicative of considerable intracellular sulfate. In addition, a strong shoulder on the low-energy side of the endogenous sulfate edge showed a form of sulfur at a slightly lower oxidation state than +6. Resolvable resonances at lower energy indicative of thiol or thioether sulfur were also observed. This work will be the subject of a separate communication.[30]

Both the cell debris and the pellet contained significant quantities of sulfur (Table I), most likely representing membrane-bound species. Average cell concentrations of these types are 0.097 M and 3.7 mM, respectively. Thus, if all these sources are combined, the *average* sulfur concentration within the blood cells of *A. ceratodes* is 0.25 M.

Vanadium was also found in significant quantity within all the various fractions obtained following lysis (Table I). From the overall quantity of vanadium found, an average concentration of 0.099 M is calculated for the blood cells of *A. ceratodes*. This value is very close to that found for the same species by Hawkins et al.[31] If ascribed purely to the vanadophores, the vanadium(III) concentration would be 1.4 M. Available evidences from EXAFS,[25] ^{1}H NMR,[24] and EPR (vide infra) spectral methods indicate strongly that virtually all the cellular vanadium (≥97%) is trivalent and nonchelated. In addition, the vanadophoric concentration of V(III) in *A. ceratodes* has been closely estimated by ^{1}H NMR[24] to be 1.3 M. Therefore, from Table I, much of the vanadium liberated on lysis must be carried into the residual solids by adsorption to insoluble proteins and membrane fragments, even in pH 2 HCl.

The total cellular [Fe] of 0.45 mM is low for this organism,[31] and this element is seen to be comparatively scarce in the plasma. Zinc was found throughout the various fractions, and the plasma level marks a significant enrichment over that in ambient seawater.

In analysis for other elements, phosphoruous was found in the cell debris (7.65 μmol) and pellet (0.2 μmol), but interestingly, not in the lysate. Evidence for significant quantities of iodine was also seen; however, the level could not be quantitated due to the formation of volatile products. Boron was found in the plasma (0.43 μmol/mL) at a level comparable to that in ambient seawater.[32] Very minor traces or no trace of Ti, Mn, Co, Ni, Mo, Cd, Pb, and Se was found.

The plasma concentration of vanadium proved particularly interesting. Ambient seawater vanadate concentrations are on the order of 39 nM[32]. Thus, vanadium levels in the plasma have apparently been enriched some 4000-fold over this. Since the plasma:lysate metal concentration ratios indicate little if any cell lysis during treatment, this result implies the possible presence of a plasma vanadium transporter. A similar suggestion may be advanced for zinc, iron, and chromium.

2. Vanadyl Ion in Vanadocytes and the Henze Solution. Figure 1a shows the room-temperature EPR spectrum of a whole blood cell suspension from *A. ceratodes*. The presence of a low molecular weight vanadyl ion species is clearly indicated. In order to further characterize the environment of the metal, the isotropic coupling constant A_0 and the corresponding g_0 value were determined and compared to those of aquo vanadyl ion in dilute H_2SO_4 and in the further presence of 0.5 M $V_2(SO_4)_3$ (i.e. 1 M V(III)). The EPR spectrum of the latter solution is given in Figure 1b. The respective magnetic data are gathered in Table II, and it may be seen that the ligation environment in each case is essentially identical,[29,33] in both liquid and frozen solutions.

In frozen solution, the vanadyl ion EPR spectra arising from whole-cell preparations proved to be broadened relative to those derived similarly from relatively dilute acidic aqueous solution (see Figure 2). This observation remained true over the several independent collections of animals, although some variation in overall line width was noted. The line width data of two such experiments are given in Table II. From the second collection group, two animals yielded sufficient blood to allow the acquisition of relatively well-resolved frozen-solution spectra. It can be seen that the line widths and magnetism shown by vanadyl ion in these single animal spectra are directly comparable to those of the packed cell sample (Table II). The EPR spectra of vanadyl ion in freshly frozen vanadocytes and in the Henze solution are shown in Figure 2, parts a and b, respectively. For comparison, the EPR spectrum of 1 mM $VOSO_4$, in 11 mM H_2SO_4 (pH 1.67) is shown in Figure 2c. The similarity in line width of the vanadocyte-derived spectrum with that of the inorganic vanadyl ion solution is noteworthy in that vanadyl ion EPR line width proved to be sensitive to $[H_2SO_4]$ (Figures 2 and 3) as discussed below. The enhanced resolution observed in the Henze solution spectrum relative to that of intact vanadocytes is significant and may reflect an increase in solution viscosity or acidity following cell lysis (vide infra).

An earlier suggestion[4,22] that the vanadyl ion arises only in aged or air-exposed cells is not tenable in this case. In our hands, fresh vanadocytes obtained from six independent collections of animals over a period of several years invariably showed a weak vanadyl EPR signal. Furthermore, since the plasma environment in vivo is aerobic, short-term exposure of aqueous cell suspensions to atmospheric oxygen during processing should not constitute a radical change of circumstance.

Since vanadocytes constitute 50–65% of the plasma cell population in *A. ceratodes*[34] with most of the remainder being signet ring cells (15–25%) and compartment cells (5–15%), the possibility exists that the observed vanadyl ion EPR signal arises from one of the latter two cell types rather than from vanadocytes. Indeed, signet ring cells (but not compartment cells) are known to contain V and S, which are stored in small electron dense plasma granules.[12] Such granules are, however, more likely to contain material in some quasi-crystalline form, possibly analogous to the iron in ferritin. However, as the room-temperature EPR spectra are clearly consistent with an aquovanadyl ion[29] rather than with a macromolecular protein complex[28,29,35–37] or quasi-crystalline solid,[29] the signet ring cell granules, as discussed above, are most likely ruled out as the source of the vanadyl ion signal.

In order to test whether the vanadyl signal could arise from residual extracellular fluid, frozen cell-free plasma supernate was examined by EPR for paramagnetism. Indeed, a very weak but discernable signal could be detected near $g = 2$. If vanadyl ion is assumed to be the source, the concentration estimated from signal intensity was <1% of that observed arising from packed vanadocytes.

The above considerations strongly support the assignment to vanadocytes of the observed EPR spectra. The concentration of vanadyl ion in vanadocytes and in the Henze solution derived from two independent collections of tunicates was quantitatied by comparative integration of frozen-solution spectra with spectra of known vanadyl ion concentration[36,38] (see Materials and

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Methods). The results, shown in Table II, indicate a low concentration of vanadyl ion in intact vanadocytes, increasing only slightly relative to total vanadium in the hemolysate. These values are somewhat greater than that observed for intact vanadocytes of *A. nigra* wherein $[VO^{2+}] = 5.7$ mM was found,[23] but less than the 60 mM concentration estimated to be present in cells from *A. mentula*.[13]

Given the method of preparing the Henze solution, and its rapid quench in liquid nitrogen, it is unreasonable to suppose that much of the freed vanadium had oxidized to vanadate or some other EPR-silent V(V) species. These results extend the precision of the earlier findings from XAS investigations, indicating minimal oxidation of V(III) in fresh Henze solution.[25] Since the experimental sample contained the pooled cells from 30–40 animals, the above concentrations can be considered as an average. It was not possible to obtain a reliable vanadyl ion concentration from the single-animal spectra.

3. EPR Line Width Determinants in Frozen $VOSO_4$ Solutions. In order to address the source of broadening of the vanadocyte vanadyl ion frozen-solution EPR signal, we decided to investigate line-broadening effects by systematic variation of the concentration of each of the pertinent intravacuolar constituents on frozen-solution EPR spectra of 1 mM $VOSO_4$ in 1 mM H_2SO_4. These constituents include acidity as H_2SO_4, counterion as SO_4^{2-}, viscosity, and high concentrations of paramagnetic transition-metal ions. The results of these experiments are in part displayed graphically in Figure 3 or gathered in Table III.

Thus, vanadyl ion EPR line width increases smoothly with concentration of vanadyl sulfate (in 1 M H_2SO_4) and varies inversely with acidity (1 mM $VOSO_4$ in H_2SO_4) (Figure 3). Interestingly, the spectrum of 1 mM $VOSO_4$ in 1 mM H_2SO_4 was a slightly more resolved, though noisier, version of that arising from 1 M $VOSO_4$ in 1 M H_2SO_4 (see figure 2, left inset). Individual lines were incompletely resolved in both spectra.

In order to test whether the observed acidity effect was an artifact of viscosity, the line width dependence on this property of 1 mM $VOSO_4$ in 1 mM H_2SO_4 was investigated. The problem of choosing an innocent additive conferring only viscosity on solutions of transition-metal ions is nontrivial. Both glycerol and poly(ethylene glycol) proved unacceptable in this regard since both gave completely resolved spectra at the lowest concentrations tested. Since these produced viscosity increases of only about 1% over pure water, the above two additives clearly exerted a specific effect, most likely complexation, on vanadyl ion. Poly(vinylpyrrolidone) (mol wt 40 000) appeared to satisfy the necessary criteria however, and the data are plotted in Figure 3 (inset). By way of comparison, the H_2SO_4 line-width data are also plotted as a function of viscosity. Inspection of the figure establishes the clear dominance of acidity over viscosity as controlling line width in low pH solutions of low to moderate viscosity.

As a further check, the effect of freezing rate on line width was examined. Solutions of 1 mM $VOSO_4$ in 1–100 mM H_2SO_4 were frozen either in liquid N_2 (freezing time $\sim$10 s) or in a freezing pentane slush ($T = -130$ °C; freezing time <1 s). No significant differences ($\Delta \leq \pm 5\%$) were found in the EPR line widths of otherwise equivalent vanadyl ion solutions frozen in either manner.

Distinctive spectra near 1380 G, arising from the forbidden $\Delta m_s = \pm 2$ transition of a possibly dimeric complex, were observed in 0.50 and 1.0 M $VOSO_4$ solutions in 1.0 M H_2SO_4 (Figure 4). At least seven lines are clearly visible in this spectrum, and additional incompletely resolved features are also apparent. However, it is not possible to decide unambiguously from these data whether the spectrum arises from a dipole- or exchange-coupled dimer (or alternatively a mixture of the two). Further experiments at liquid-He temperature to resolve this question are anticipated. Significantly enhanced resolution was observed when dilute solutions yielding otherwise broadened spectra were made stepwise more viscous with poly(vinylpyrrolidone) (PVP) as shown in Figure 3 (inset). Therefore, spectral broadening is likely due to the formation and trapping of a magnetically coupled, probably binuclear vanadyl species, more than one of which may be present. This point is amplified below.

The response of vanadyl ion EPR line width to serially increasing sulfate concentration proved to be rather complicated (Figure 3). Sequential additions of sulfate up to 10 mM, added as Na_2SO_4, produced a succession of more highly resolved vanadyl ion spectra, which were also increasingly smooth in appearance. At 15 mM added Na_2SO_4, a transition occurred, after which the spectra appeared to be a convolution of two superposed vanadyl ion spectra, one relatively sharp and the other very broad. The relative intensity of the broad underlying signal increased at the expense of the more highly resolved one through 30 mM added Na_2SO_4 (Figure 5). Above this concentration, the broad component diminished in relative intensity, becoming small but still noticeable at 500 mM Na_2SO_4. The pH of these solutions ranged from 2.65 (zero added Na_2SO_4) to 3.48 (1000 mM added Na_2SO_4). It should be noted that the latter pH is still sufficiently low to inhibit both air oxidation of VO^{2+} [36] and dimerization of this ion to $(VOOH)_2^{2+}$ [40] or other EPR-silent oligomeric species.[27,29]

A variation in signal line width of about ±5% was noted from time to time in otherwise similarly composed Na_2SO_4 series samples. This appeared to reflect variation in frozen-solution composition, possibly dependent on freezing history. Indeed line width increases were noted when single samples were thawed and refrozen: that of the 20 mM Na_2SO_4 solution increasing from 44 to 54 G on thawing and refreezing, for example. Accordingly the Na_2SO_4 series line widths are those observed on initial freezing and comprise two independent data sets. In contrast, frozen-solution EPR spectra of $VOSO_4$ in 1 M H_2SO_4 were essentially insensitive to freeze/thaw cycling.

When 50 mM NaCl or $NaClO_4$ was substituted for 50 mM Na_2SO_4 added, 1 mM $VOSO_4$ in 1 mM H_2SO_4 solutions exhibited well-resolved frozen-solution EPR spectra (Table III). Furthermore, the spectra appeared homogeneous, with no evidence of the broad, underlying component as noted above with sulfate.

Finally, the effect of high concentrations of a paramagnetic metal ion on vanadyl ion EPR line width was examined (Table III). Cobalt(II) was chosen for systematic study because its electron spin relaxation time is similar to that of V(III).[41,42] Furthermore, the dipolar coupling of electron spins between $Co^{II}(aq)$ and $VO^{2+}(aq)$ should not be complicated by electron self-exchange such as would undoubtedly occur between V(III) and VO^{2+} in acid solution. Of the various solutions containing $CoSO_4$, only that also 500 mM in H_2SO_4 was sufficiently resolved to measure the width of the $(-7/2)_\parallel$ line. Of the remainder, those containing 200 and 100 mM H_2SO_4 yielded an estimate of this line width. The rest were unresolved. The partially resolved spectra again appeared to consist of superposed broad and sharp components, the former persisting over the entire range of acidity. In that sense, these spectra were similar to, but not identical with, the $VOSO_4/Na_2SO_4$ spectra described above. When 500 mM $CoCl_2$ was substituted for $CoSO_4$, the EPR spectrum of $VOSO_4$ was well resolved and homogeneous, even at relatively lower acidity (Table III). Thus the contribution to line broadening from the aquo Co(II) ion in these spectra must be small. That is, since 0.5 M sulfate should exert the same screening effect as 1 M chloride by the ionic strength criterion, the data again support the idea of a specific sulfate-mediated broadening mechanism.

Previously Albanese and Chasteen[43] ascribed the residual EPR line width observed from vanadyl ion in frozen-glycerol glasses to unresolved proton/electron superhyperfine interactions. However, this effect is only a few gauss and cannot account for the observed broadening reported here.

Broadened EPR spectra noted to result from freezing solutions of cupric ion[44] have been ascribed to the superposition of spectra

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arising from various metallic species including clusters.

In order to elucidate the source of sulfate-mediated line broadening, this effect was analyzed in some detail. As mentioned above, in frozen, concentrated, acidic VO^{2+} solutions, characteristic $g = 4$ spectra were observed near 1380 G, indicative of a forbidden $\Delta m_s = \pm 2$ transition within a possibly binuclear vanadyl complex. The experiments with PVP indicated that similar dimers could be the source of the broadening in dilute solution as well. However, at 1 mM vanadyl ion, frozen-solution EPR spectra were too noisy to observe $g = 4$ transitions in the sulfate-broadened spectra.

According to Molin et al.,[45] Zamaraev,[45,46] and Nikitaev[46] line broadening in liquid vanadyl ion solutions can arise due to spin exchange within short lived vanadyl dimers. Such dimers were adduced to reactions 1–3, with discrete $VOSO_4$ complexes participating in dimer formation, and wherein bridging groups and liganding water are left unspecified. In contrast to sulfate, it was found that Cl^-, ClO_4^-, and NO_3^- promote dipolar spin coupling only through Coulombic charge screening in short-lived termolecular collision complexes.

$$VO^{2+} + VO^{2+} = [V_2O_2]^{4+} \quad (1)$$

$$VOSO_4 + VO^{2+} = [V_2O_2SO_4]^{2+} \quad (2)$$

$$VOSO_4 + VOSO_4 = [V_2O_2(SO_4)_2] \quad (3)$$

From the equilibrium data of Strehlow and Wendt,[47] the concentrations of $VO(H_2O)_5^{2+}$, $VO(H_2O)_5SO_4$ and $VO(H_2O)_4SO_4$, (i.e. the free aquo ion, the tight ion pair, and the aquovanadyl sulfate complex, respectively) were calculated for the system 1 mM $VOSO_4$ in 1 mM H_2SO_4 with increasing $[SO_4^{2-}]$. The contribution from $VO(SO_4)_2^{2-}$ was neglected. The result of these calculations is presented graphically in Figure 6. If reactions 1–3 were responsible for the sulfate-mediated vanadyl ion EPR line broadening, then a square law dependence of line broadening on concentration should be observed. The results of this analysis are given in the Figure 6 inset. Here the tight ion pair and the sulfate complex were treated as equivalent neutral species. It can be seen that the square dependence of reactions 1 and 2 show behavior qualitatively similar to the EPR line broadening trend, particularly in the region bounded by 10–100 mM added sulfate. That is, a smooth fall off in tetrapositive dimer formation from reaction 1 is predicted with increasing sulfate concentration. In contrast, the term arising from reaction 2 traverses a maximum between 5 and 50 mM added sulfate, and dominates the reaction 1 term above 10 mM sulfate. The slow decrease of the reaction 2 term above 50 mM sulfate accounts for the observed persistence of the broadline spectral component even to high sulfate concentrations. If this analysis is correct, the data also imply that the sulfate-mediated dipositive dimer (reaction 2) is magnetically distinct from the tetrapositive dimer (reaction 1), since it is the added presence of the former that apparently produces the non homogeneously broadened spectra.

The further increase in line width predicted to continue beyond 100 mM Na_2SO_4 by the reaction 3 term indicates its relative unimportance, especially at high sulfate concentrations where anionic screening effects are expected to predominate.[44-46] This latter notion is supported by the well-resolved, homogeneous spectra noted when 50 mM NaCl or $NaClO_4$ was substituted for Na_2SO_4 (Table III).

The significance of this analysis, if correct, implies the formation of discrete vanadyl ion dimers in liquid solution. The lifetime of these complexes is likely small, since little or no variation in *liquid* 1 mM $VOSO_4$ solution EPR line width was observed over the concentration range of 0.1–2.5% PVP in 1 mM H_2SO_4 or over the acidity range 1–100 mM H_2SO_4. Most likely, the dimeric species are trapped, upon solution freezing, in approximate proportion to the liquid-solution composition. It is also likely that at least two such dimers are present in solutions containing sulfate and moderate acidity (pH 2.5–3.5). A more exact understanding of, e.g., their structure must await further experimentation.

4. Intracellular pH in Vanadocytes. Several lines of evidence can now be brought to bear on the problem of intracellular pH in the vanadocytes from *A. ceratodes*. Indeed, the following considerations most likely apply strictly to the *intravanadophoric* milieu since there is no reason to believe that the cellular cytoplasm is in any way unusual.[1]

The EPR results reported here are definitive in establishing the presence of $VO(H_2O)_5^{2+}$ in vanadocytes in both liquid and frozen solution. This fact alone requires that the surrounding medium be acidic, since no EPR signal from this ion is observed at pH $\geq$4.9. At neutral pHs, the chemistry of vanadyl ion is dominated by the formation of a variety of EPR-silent[27,29] oligomers.[27,29,40,48,49] Viscous control of vanadyl ion EPR line width in otherwise internally neutral vanadocytes can, therefore, be discounted since the vanadophoric constituents are undoubtedly at thermodynamic equilibrium. Although the observed vandophoric vanadyl ion EPR line width could be produced by a combination of mild acidity (pH 3.5) along with an appropriate viscosity, considerations discussed below militate against this possibility. In any case, from the observed dependence on acidity of both the appearance and line width of the aquo vanadyl ion EPR signal, it is clear that the vanadophoric contents must be in an acidic regime.

Further restrictions on pH are imposed by previous ^1H NMR studies[24,50] of whole-cell preparations from *A. ceratodes*, which demonstrated the presence of four to five ligating waters in the first coordination shell of the intracellular V(III). Such a finding is consistent *only* with an acidic pH, since extensive hydrolysis of the aquo ion above pH 3 would undoubtedly produce oligomeric vanadium(III) hydrous oxides.

In addition, from the data given by Newton and Baker,[51] a dimerization constant of $2.35 \pm 0.18 \times 10^{-5}$ M can be calculated for the reaction $2V^{3+} + H_2O = (VOV)^{4+} + 2H^+$, neglecting the water term. From this, at e.g. pH 4.0, virtually all the V^{3+} in aqueous solution would reside in the tetrapositive dimer, probably as the sulfate complex.[52,53] At pH 2.0, 28% would be so represented. In the XAS studies referred to above,[25] the presence of a significant concentration of oxide- or hydroxide-bridged V(III) dimers would have been clearly visible as enhanced EXAFS scattering amplitude along the distance coordinate in k-space. No such effect was observed. Thus the concurrent attributes of the observed presence of aquovanadium(III) ion, along with the lack of any observed dimer strongly argue for a vanadophoric pH $\leq$2. If this is combined with the EXAFS results specifying monomeric V(III) surrounded by six oxygens at 1.99 Å (and including no sign of an ordered chelate-imposed second atom shell) along with the ^1H NMR result of four to five ligating waters, the most reasonable form to be deduced for vanadophoric vanadium(III) in the presence of high $[SO_4^{2-}]$ is within a $V(SO_4)(H_2O)_{4-5}^+$ complex ion.

With respect to this suggestion, the recent structural elucidation using NMR and mass spectrometry[55] of one of the yellow tunicate blood pigments, termed tunichromes, from *A. nigra* is particularly relevant. These compounds, present in vanadophores at $\sim$1 M concentration, are now known to include a tripod of pyrogallol subunits, potentially conveying an octahedral array of six ligating oxygens to a transition-metal center. The possibility that this molecule might serve as a chelating ligand for intracellular V(III) led us to reexamine the EXAFS spectra, along with the corresponding Fourier transforms, for V(III) in living vanadocytes from *A. ceratodes* and for $V(catecholate)_3^{3-}$. The latter compound is

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likely to be a good model for any tunichrome–V(III) complex. The single-frequency EXAFS spectrum and single symmetrical Fourier transform peak observed for the vanadocyte vanadium data[25] contrasted perceptibly with those of the $V(catecholate)_3^{3-}$. The spectrum of the catecholate complex showed a distinct beat frequency component,[26] which can only arise from more than one coordinating sphere of atoms. The corresponding Fourier transform of this spectrum exhibited a well-resolved second-shell peak, providing a clear indication of the ordered array of phenolic carbon atoms at ca. 2.89 Å from V(III). The absence of the second frequency component in the vanadocyte EXAFS data is strong evidence for the lack of a highly ordered chelate shell about intracellular V(III). This conclusion could be further strengthened by studying the EXAFS as a function of temperature down to 4 K.

It remains to show that the vanadyl ion line width observed in the frozen-solution EPR spectrum of vanadocytes accurately reflects the internal vanadophoric pH in the presence of high [V(III)]. The effect of Co(II) and high $[VO^{2+}]$ on vanadyl ion EPR line width indicate that very significant broadening can be caused by the combination of sulfate with paramagnetic aquo ions even at appreciable (and biologically unlikely) acidities. Therefore, the relatively narrow line widths observed in the EPR spectrum from vanadocytes (Table II) can arise only if the opportunity for vanadyl/vanadyl and V(III)/vanadyl spin-exchange interactions is restricted.

In order to test this hypothesis, a solution of 2 mM vanadyl ion with 0.5 M $V_2(SO_4)_3$ in 15 mM H_2SO_4 (solution pH 1.9) was prepared. The EPR spectrum of this frozen solution is displayed in Figure 7 and shows a $(-7/2)_{\parallel}$ EPR line width of 31 G (Table III). This spectrum is clearly more highly resolved than that of the analogous $CoSO_4$ solution (cf. Table III). From Figure 3 the predicted EPR line width of vanadyl ion in frozen 15 mM H_2SO_4 (liquid-solution pH ~1.57) is 21 G, 10 G less than was observed. As discussed above, V(III) in vanadocytes has at least four of six sites open to aquation, this increasing to five of six for 0.4 M V(III) in 0.17 M H_2SO_4.[50] Therefore, at any physiologically reasonable acidic pH, concentrated aqueous solutions of V(III) sulfate will most likely contain a considerable amount of $(VSO_4)^+$ complex ion.[52,53] Since the estimated $[SO_4^{2-}]_{free}$ in both vanadocytes and the 1.0 M V(III) sulfate solution should be similar (and relatively small), the effect of this anion on the respective vanadyl ion EPR line widths should also be comparable. Furthermore, inspection of the pertinent $A_{\parallel}$ values in Table II supports estimation of a similar equatorial ligand field about the VO^{2+} ion in the two inorganic milieus and in vanadophores. In any case, since no evidence of a superposition of broad and narrow lines is observed in the Figure 7 spectrum (compare Figure 5), the formation and presence of sulfate-mediated vanadyl dimers can be discounted.

The measured pH of the above inorganic V(III)/VO^{2+} solution was only 1.9, reflecting the buffering effect of the excess sulfate present. From Figure 3, the observed 31-G line width is the same as that of 1 mM $VOSO_4$ in 6 mM H_2SO_4, which also exhibited a pH of 1.9. It therefore appears that the V(III) present has exerted little if any paramagnetic broadening effect on the EPR spectrum of vanadyl ion. Rather, the most parsimonious interpretation[54] is that pH is the determinant of line width in such solutions.

Thus from Figure 3, the 34–24 G line widths observed arising from vanadyl ion in *A. ceratodes* vanadocytes most likely reflect an internal pH near that of vanadyl ion solutions containing 6–11 mM H_2SO_4. The measured pH of these solutions were 1.9 and 1.7, respectively. Given the buffering effect of the excess sulfate present, in analogy with the above inorganic $V_2(SO_4)_3$ solution, the average acidity as H_2SO_4 within the vanadophore can be estimated to be near 15–18 mM. The corresponding average internal pH of 1.8 ± 0.1 is just below that required to prevent significant hydrolysis of $V(H_2O)_6^{3+}$ to $HOV(H_2O)_5^{2+}$ [36] or $(VOV)^{4+}$ [51] (see above) and is just sufficient to inhibit autoxidation ($E_{pH\,1.8}$ = 283 mV for the V(III)/VO^{2+} couple).

This level of intracellular acidity is not unprecedented among marine organisms. Two species of algae, *Desmarestia ligulata* var. *ligulata* and *Desmarestia viridis*, are known to accumulate H_2SO_4 in vacuoles until an average internal pH of 0.5–0.8 is reached.[56] Interestingly, the vacuole is impermeable to reverse transport of internalized sulfate. Assays of *Desmarestia firma*, a closely related species, revealed only minor traces of vanadium (1.5–2.5 ppm).[57] Additionally, vacuolated cells in the blood and in the test of certain tunicates[1,58,59] are known to contain acid (pH <3) in the absence of vanadium.

The preceeding analysis can now be applied, with reservations (see below), to extant data pertaining to other tunicate species. An intracellular pH of ca. 1.8 is consistent with the vanadocyte-derived EPR spectrum published by Swinehart et al.[4] for the vanadophoric contents of *Eudistoma diaphanes*. This is a colonial tunicate from the order Aplousobranchia and is thus quite removed evolutionarily from the solitary *A. ceratodes* (Phlebobranchia).

The frozen-solution EPR spectrum obtained from *A. nigra*, shown by Dingley et al.,[23] contains insufficient detail to gain a good estimate of pH, although the overall spectral line shape appears consistent with a range of pH 1.9–2.3. However, the ambient-temperature EPR spectral line shape is broadened compared to that arising from *A. ceratodes*. In addition, doubling of some of the EPR lines appears to indicate[3] that VO^{2+} senses two slightly different environments in blood cells from this organism. The isotropic coupling constant, A_0, can be estimated to be 1.06×10^{-2} cm^{-1} from the latter spectrum, consistent with the presence of $VO(H_2O)_5^{2+}$. However, the corresponding g_0 is 1.937, an unusual value possibly indicative of the apparent mixture of magnetic environments. Therefore the frozen-solution line width is most likely not cleanly responsive to pH in this case. Indeed, it should be stressed that it is necessary to demonstrate the exclusive (or cleanly resolvable) presence of aquovanadyl ion before the line-width criterion for pH determination can be applied.

The above levels of intravanadophoric acidity are very near those found within cells from *A. mentulla* (pH 1.8) and *A. aspersa* (pH 1.0) by Bell et al.[13] However, this coincidence of values may be fortuitous, since the reported experimental protocol of cell lysis and pH measurement was apparently not done anaerobically. In this eventuality, hydrolytic autoxidation of freed V(III) could liberate significant acidity. Since the herein described work is noninvasive, such problems are circumvented.

The pH within vanadocytes from *A. nigra* has been probed by following $^{14}CH_3NH_2$ diffusion into these cells.[14] A neutral pH was determined to characterize vanadocytes, and this finding was interpreted to imply a neutral pH within vanadophores. However, the method used is beset by the problem that any diffusant must pass through the vanadocyte cytoplasm intermediate between exogenous solution and vanadophore. Since the vanadocyte is some 14 times the aggregate volume of the vanadophores,[24] significant buffering effects may occur. Indeed, only an average pH would be determinable by this method since the whole cell is being sampled.

The acidity contained within *A. ceratodes* vanadocytes has also been recently examined by Hawkins et al.[31] Three methods to access this information were employed, viz. (i) intracellular diffusion of pH indicators, (ii) ^{31}P NMR on whole-cell preparations, following the correlation of phosphate chemical shift with pH, and (iii) pH measurement of anaerobically lysed cell samples. The first two methods yielded the contradictory results of pH 4.6–5.2 and pH 6.9, respectively. However, the problems attendent to the use of indicators, especially in the presence of reducing agents, has been previously pointed out.[3] In addition as we have shown, only the lower region of the indicator pH range is consistent with the observation of an EPR signal from aquovanadyl ion.

In the NMR experiment, three broadened ^{31}P resonances were observed, two of which were displaced well out of the pH-induced chemical shift range apparently by paramagnetic contact inter-

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action with V(III). The authors correctly point out that, in the event, assignment of any resonance position as reflecting strictly pH is problematic. With regard to the in-range peak, if taken to reflect acidity, the derived pH 6.9 is a reasonable value for the cytoplasm of the vanadocyte, which could easily contribute a phosphate resonance to the whole-cell experiment. In any case, a vanadophoric pH 6.9 is clearly inconsistent with other results presented and discussed herein.

From the anaerobic lysis experiments (iii, above), Hawkins and co-workers attempted to account for the total acidity liberated as arising from a combination of the internal acidity originally present, augmented by the acid generated following lyolytic hydrolysis of trivalent vanadium and iron released from the vanadophore. However, the total acid produced (3.15×10^{-5} mol from 0.5 mL of packed cells) is so much greater than that which was estimated to be caused by metal hydrolysis (1.3×10^{-5} mol) that the remainder back-calculates to a vanadophoric concentration[24] of 0.28 M H_2SO_4. This is most likely an unrealistic figure, and indeed the authors suggested that the extra acidity may derive from the release of protons when V(III) is chelated following cell lysis. The analytical results presented here support this latter possibility since much of the liberated vanadium was found bound to cellular debris (Table I).

In any case, among Phlebobranchs, and possibly Aplousobranchs, at least one of the vanadium-accumulating tunicates appears to maintain this metal ion at a pH less than 2. Significantly, this pH is sufficient to convert absorbed oceanic orthovanadate ion completely to the monopositive VO_2^+ ion.[36] Thus these data stand in support of the acidic reduction mechanism of vanadium accumulation by tunicates proposed by Macara et al.[60]

The small concentration of vanadyl ion found within the vanadocyte seems to militate against any important functional role for this ion in *A. ceratodes*. It seems more reasonable to suppose that it represents vanadium only partially reduced from ingested vanadate and trapped as vanadyl ion when the cells were frozen. A similar hypothesis has been made for vanadyl ion in *A. nigra* following vanadate uptake studies.[3] If true, then vanadyl ion concentration within the vanadocyte would be a complex function of feeding rate and oceanic vanadate concentration. It would, therefore, be expected to fluctuate about some small value, as has been observed.

The utility of V(III) to tunicates remains an unresolved question; however, any hypothesis must explicitly take note of the low oxidation state in which this ion is maintained.[24] The contents of vanadophores are now known to comprise a surprisingly complex mixture, with 1.4 M V(III), ~1 M tunichromes, 1.3 M sulfate, ~0.85 M complex sulfur of possibly novel types, plus other metals, and possibly other constituents stored in a low-pH solution. How these materials contribute to the fitness with which tunicates respond to environmental stress is likely to be found in the functional role of the vanadophore, e.g. in the production of test, in the immune response, or as a defense against surface fouling, among others. It is, therefore, not necessary inter alia to restrict the functional significance of any particular vanadophoric constituent either to the interior of the vanadophore or to interaction with a second vanadophoric constituent. In that regard, we suggest[50] that V(III) might produce peroxide-like species following exposure to the aerobic, mildly alkaline[61,62] intertidal environment wherein these animals are found. Such reagents, if produced on site by vanadocyte lysis subsequent to minor injury,[63,64] might forestall bacterial invasion, as well as possibly seal off such injuries by sclerotization of the surrounding tissue. This suggestion is consistent with the known oxidative chemistry of, e.g., peroxovanadates,[65] as well as the wound-healing activity of vanadocytes.

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Registry No. S, 7704-34-9; V, 7440-62-2; Fe, 7439-89-6; Zn, 7440-66-6; Cr, 7440-47-3; Cu, 7440-50-8; VO^{2+}, 20644-97-7; SO_4^{2-}, 14808-79-8; Co^{2+}, 22541-53-3; V^{3+}, 22541-77-1; Cl^-, 16887-00-6; ClO_4, 14797-73-0; H_2SO_4, 7664-93-9; $VO(H_2O)_5^{2+}$, 15391-95-4; P, 7723-14-0.

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σ-Bonded Organochromium(III) Complexes. 3. Decomposition in Acid Solution of Chromium(III) Complexes Containing Pyridylmethyl and Polydentate Amine Ligands[1,2]

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The decomposition of σ-bonded (pyridylmethyl)chromium complexes, 2- and 3-$NC_5H_4CH_2CrL_n$ (L = dap (1,3-diaminopropane), dien (diethylenetriamine), trien (triethylenetetramine), and [15]aneN$_4$ (tetraazacyclopentadecane)), was investigated in aqueous perchloric acid under aerobic conditions. Except for L = 15[ane]N$_4$, Cr–C bond scission was preceded by complete aquation in the case of the 2-isomers and partial aquation for the 3-isomers. The aquation rates were compared with those of inorganic chromium complexes containing similar amine ligands. Kinetic data for the Cr–C bond cleavage were correlated with those for the analogous ethylenediamine (en) and aquo (H_2O) systems. The activation parameters and product studies are in support of a homolytic pathway for the Cr–C bond cleavage.

Reactions of organochromium complexes such as hydrolysis, protonolysis, oxidation, and polymerization and reactions with $HgCl_2$, $TlCl_3$, halogens, and organic halides have received considerable attention[3-5] and form the subject of a recent review.[6] In particular, the mechanistic aspects of the chromium–carbon

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