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Further Investigation of the Status of Acidity and Vanadium in the Blood Cells of *Ascidia ceratodes*

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Vanadium-containing blood cells from the tunicate *Ascidia ceratodes* have been subjected to further study with respect to intracellular acidity and magnetism. Anaerobic lysis of two independent packed whole blood cell samples released acid to pH 1.4 and pH 1.1, respectively. EPR spectra of two frozen Henze solution samples, obtained from lysing cells packed in separate quartz EPR tubes, yielded similar values based on the width of the $-7/2_{\parallel}$ line of the released pentaaquovanadyl ion. Whole blood EPR spectra acquired at ambient temperature ($A_0 = 1.078 \times 10^{-2}$ cm^{-1}, $g_0 = 1.967$) and at liquid-nitrogen temperature from rapid-freeze samples ($A_{\parallel} = 1.833 \times 10^{-2}$ cm^{-1}, $A_{\perp} = 0.7188 \times 10^{-2}$ cm^{-1}, $g_{\parallel} = 1.933$, $g_{\perp} = 1.992$) were consistent with the presence of endogenous pentaaquovanadyl ion. The 28 G line width of the $-7/2_{\parallel}$ EPR line in the frozen-solution spectrum indicated a pH of 1.8 for the intracellular environment of this ion. In contrast to whole blood, the EPR spectrum of a frozen-tissue sample revealed vanadyl ion within a mixture of ligand environments. ^{1}H NMR experiments at 100 MHz on packed samples of whole blood showed a broad resonance 21.5 ppm downfield from TMS, in addition to the bulk intracellular water peak at 4.5 ppm. Line-shape analysis of the 21.5 ppm signal, visualized by selective radio-frequency power saturation of the 4.5 ppm peak, indicated a nearly pure Gaussian envelope. These results are discussed in terms of our previous experiments, here corroborated, as well as the subsequent experiments reported by Brand, Hawkins, and Parry, who did not observe acidity on cell lysis and were unable to obtain EPR spectra of the pentaaquovanadyl ion or to obtain ^{1}H NMR spectra showing the Gaussian resonance at 21.5 ppm, using blood cells obtained from *A. ceratodes* and other tunicates. An experimental basis for this apparent contradiction is discussed. Detailed line-shape analysis of published and previously unpublished ^{1}H NMR spectra of packed intact blood cells from *A. ceratodes* is presented.

In a recent publication[1] we described a method wherein the line width of the $-7/2_{\parallel}$ EPR signal from pentaaquovanadyl ion could be used to measure pH over the range $2.6 \geq \text{pH} \geq 0.8$. The method was applied to detrmine the acidity within the vanadium-containing vacuoles of blood cells from the tunicate *Ascidia ceratodes*, yielding a pH of 1.8 ± 0.1. This finding is consistent with previous results from X-ray absorption spectroscopy (XAS) measurements, which indicated that nearly all the vanadium in these cells is present in the form of unchelated V(III).[2] The vanadium(III) is, however, liganded by approximately four exchangeable waters, which contribute a broad, Gaussian resonance at 21.5 ppm downfield from TMS in the ^{1}H NMR spectrum of packed whole blood cells from *A. ceratodes*[3] or *Ascidia nigra*.[4] Large amounts of intracellular sulfate have also been detected, both by chemical means[1,3] and by XAS measurements,[5,6] implying that the V(III) is present chiefly as the $V(SO_4)(H_2O)_{4-5}^+$ complex ion within the vanadium-containing blood cells from *A. ceratodes*.

However all these results have more recently been disputed.[7] Hawkins and co-workers have presented data indicating the lack of acidity in blood cells from *Phallusia julinea*, since anaerobic lysis of packed cell samples yielded a mixture with neutral pH. Following magnetic resonance experiments, they have also questioned the presence of either pentaaquovanadyl ion or aquovanadium(III) sulfate within ascidian blood cells. On the strength of these findings our earlier EPR and ^{1}H NMR results referred to above were rendered suspect; the 21 ppm ^{1}H NMR band being ascribed by Hawkins et al. to an instrumental phasing error.

We present here data from new lysis and EPR experiments and further ^{1}H NMR results that are completely consistent with the earlier conclusions[1-3] regarding vanadium and acidity within blood cells from *Ascidia ceratodes*. In addition, an experimental basis for the apparently conflicting results described in ref 7 is discussed.

Materials and Methods

Specimens of *A. ceratodes* were obtained and the blood removed as described previously.[1,3,6] For anaerobic lysis, two independent blood samples were centrifuged at 500*g* for 5 min yielding 0.14 and 0.19 cm^3 of packed cells, respectively. The supernatant was removed under argon inside a thoroughly purged glovebag and the volume made to 1.0 cm^3 with dinitrogen-saturated deoxygenated (3× freeze/pump/thaw) doubly deionized water. Lysis was accomplished by bubbling the mixture carefully with a dinitrogen stream along with gentle stirring with the tip of the gas delivery pipet. The dinitrogen stream was assessed to be dioxygen-free by its inability to decolorize a dilute aqueous solution of methylviologen radical cation after 3 h of bubbling.

Anaerobic pH measurements on whole blood lysates were made within the glovebag by using an MI-410 micro combination pH probe (Microelectrodes Inc., Londonderry NH, 03053) attached to an external Beckmann Model 3500 pH meter.

Room-temperature EPR measurements[8] were carried out on the combined plasma of two *A. ceratodes* specimens. Spectral data were obtained on this sample within 15 min of removal from the organisms. A thin-walled glass capillary was used to contain the cells, and the sample was kept on ice prior to measurement. No evidence of cell lysis was observed (formation of blue or brown specks) either before or after EPR spectral acquisition.

For the rapid-freeze EPR experiments, blood taken from three individual animals was, immediately upon removal, or after 5 or 30 min, injected through a polypropylene tube directly into the bottom of a quartz EPR tube immersed in a freezing pentane slush (−130 °C).[9] The samples froze immediately upon contact with the quartz tube walls.

The tissue sample from a specimen of *A. ceratodes* was obtained by flash freezing a living animal through rapid immersion in a freezing pentane slush.[10] The frozen animal was then ground to a fine granular consistency under a cold pentane blanket. A sample of the resulting material was placed into a cold quartz EPR tube. Frozen samples of whole blood or tissue were maintained within EPR tubes submerged in a liquid-nitrogen storage Dewar prior to spectral measurement.

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(1) Frank, P.; Carlson, R. M. K.; Hodgson, K. O. *Inorg. Chem.* **1986**, *25*, 470.
(2) Tullius, T. D.; Gillum, W. O.; Carlson, R. M. K.; Hodgson, K. O. *J. Am. Chem. Soc.* **1980**, *102*, 5670.
(3) Carlson, R. M. K. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 2217.
(4) Kustin, K.; Levine, D. S.; McLeod, G. C.; Curby, W. A. *Biol. Bull. (Woods Hole, Mass.)* **1976**, *150*, 426.
(5) 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. *Nucl. Instrum. Methods Phys. Res., Sect. A* **1986**, *246*, 797.
(6) Frank, P.; Hedman, B.; Carlson, R. M. K.; Tyson, T. A.; Roe, A. L.; Hodgson, K. O. *Biochemistry* **1987**, *26*, 4975.
(7) Brand, G. S.; Hawkins, C. J.; Parry, D. L. *Inorg. Chem.* **1987**, *26*, 627.
(8) EPR experiments were carried out by using a Varian E-112 Century Series spectrometer operating in the X-band.
(9) Freezing pentane slushes have found use in rapid-freeze EPR experiments wherein samples are frozen within milliseconds (cf. Reinhammar, B.; Oda, Y. J. *J. Inorg. Biochem.* **1979**, *11*, 115).
(10) The organism froze immediately upon immersion, and the granulated material was at no time thawed prior to spectral acquisition.

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^{1}H NMR measurements were carried out as described previously;[3] details are given in the captions to Figures 2 and 3.

Results and Discussion

To support their assertion that a neutral pH exists inside the vacuoles of vanadium-containing blood cells, Hawkins et al.[7] cite recently reported experiments in which (1) the distribution of ^{14}C-labeled methylamine[11] and 9-aminoacridine[12] between extracellular buffers and cytoplasm, (2) ^{31}P NMR chemical shift of inorganic phosphate,[11,13] or (3) infusion of pH indicators[13,14] were used to study intact ascidian blood cells. These results have been discussed extensively by ourselves[1] and others.[12,13] None have yielded a robust determination of intravanadophoric pH as applied to ascidians. However it is worth reiterating here that the blood chemistry of tunicates is highly varied. For example the blood chemistry of *Boltenia ovifera*, the organism studied by using 9-aminoacridine,[12] is dominated by iron. Therefore findings relating to such species cannot be considered rigorously applicable to the question of intravacuolar pH values in *A. ceratodes*.

Lysis. Anaerobic lysis (see Materials and Methods) of each of two blood cell pack samples (0.14 and 0.19 cm^3, respectively) was accompanied by considerable frothing, indicative of the release of intracellular protein and lipids. Within a few seconds, the usual red-brown Henze solution[15] was obtained. This is in keeping with the finding that formation of the Henze solution need not be accompanied by oxidation of V(III).[1,2] The final stable pH values, measured after several minutes of N_2 bubbling and stirring was pH 1.4 (for 0.14 cm^3 of cells) and pH 1.1 (for 0.19 cm^3 of cells). These acidities are consistent with those reported by Swinehart and co-workers,[13] also on anaerobic lysis of *A. ceratodes* blood cell packs, but in contrast with the findings of Hawkins et al. for blood cells from *P. julinea*.[7]

The above pH values were not used to back-calculate intracellular acidity because of the inhomogeneity of the samples after lysis. The observed acidities are in any case more likely indicative of the artifactual ligation of the liberated V(III) by proteins or smaller complexing molecules, or by hydrolysis of this ion, than of the original acid present.[13]

In this regard, earlier EPR studies[1] on two independent Henze solutions, prepared in situ by thawing and refreezing a frozen blood cell pack within the EPR tube, yielded EPR spectral data indicating a small increase in vanadyl ion concentration,[16] in conjunction with significantly sharpened lines. Since the magnetism of the vanadyl ion both before and after lysis was that of the pentaaquo ion, the observed sharpening does not reflect a change of state for the vanadyl ion. Therefore, in the absence of a large increase in viscosity, the width of the $-7/2_{\parallel}$ line can be used to ascertain the pH of these solutions.[1]

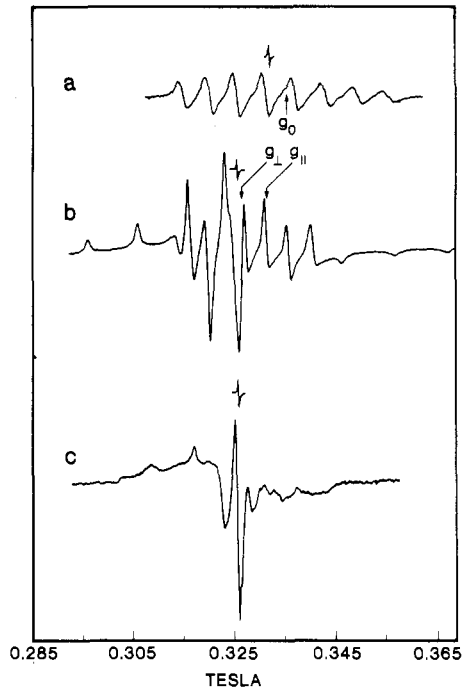

Figure 1. EPR spectra of vanadyl ion within *A. ceratodes*. (a) Spectrum of whole blood combined from two animals. The spectrum was taken within 15 min of removal, at ambient temperature. Power was 10 mW, gain 8 × 10^3, modulation 8 G, and frequency 9.550 GHz; (b) Spectrum of a rapid-frozen sample of whole blood, taken from a single animal. Temperature was near LN_2, power 20 mW, gain 5 × 10^3, modulation 8 G, and frequency 9.188 GHz. (c) Spectrum of a sample of granular frozen tissue (see Materials and Methods). Temperature was near LN_2, power 25 mW, gain 1.25 × 10^4, modulation 8 G, and frequency 9.190 GHz. The pitch signal marks (a) 3406.0 (b) 3297.3, and (c) 3270.0 G.

For the two independent Henze solution preparations, lysis increased the average [VO^{2+}] from 0.9 to 5.5 mM and from 2.6 to 8.7 mM. The corresponding EPR spectra exhibited decreases in the $-7/2_{\parallel}$ line width of 7 G (24.3 to 18.3 G) and 15.5 G (34.0 to 18.4 G), respectively. These changes imply drops in pH from 1.7 to 1.4 and from 1.9 to 1.4, respectively. The consistency of the EPR results with the measured pH of the anaerobic lysates described above is suggestive that vanadyl ion senses no radical increase in viscosity on Henze solution formation.

Oxidation of V(III) to VO^{2+} by any dioxygen present during lysis yields a net of one proton per metal ion. Therefore the acid liberated from the above production of vanadyl ion, combined with the acid already present, can only yield pH values of 2.2 in each case, i.e. less than 20% of the acid actually observed. The remainder must then derive from protons liberated either through complexation of the released V(III) by newly available proteins or other complexing molecules[17] or by hydrolysis of this ion following its admixture with the previously neutral cytosol[1,13,16] or from cells that contain acid, but no vanadium.[14]

EPR Studies. Figure 1a shows the room-temperature EPR spectrum of combined whole blood from two individual *A. ceratodes* specimens. This spectrum was acquired within 15 min of removal from the healthy, living animals. The spectrometer settings (cf. figure caption) are consistent with the presence of a significant and readily observable vanadyl ion concentration. The magnetism of this ion (A_0 = 1.078 × 10^{-2} cm^{-1}, g_0 = 1.967) identifies it as the pentaaquovanadyl ion, i.e. $VO(H_2O)_5^{2+}$, in

(11) Dingley, A. L.; Kustin, K.; Macara, I. G.; McLeod, G. C.; Roberts, M. F. *Biochem. Biophys. Acta* **1982**, *720*, 384.
(12) Agudelo, M. I.; Kustin, K.; McLeod, G. C. *Comp. Biochem. Physiol. A* **1983**, *75A*, 211.
(13) Hawkins, C. J.; Gweneth, A. J.; Parry, D. L.; Swinehart, J. H.; Wood, A. L. *Comp. Biochem. Physiol. B: Comp. Biochem.* **1983**, *76B*, 559.
(14) Hawkins et al.[7] make the claim that electron microscopic studies concluded that vanadium is predominantly present in an insoluble matrix, citing work by Kalk,[38] Pirie and Bell,[24] and a private communication. This conclusion appears nowhere in ref 24. Instead Pirie and Bell find that vanadocyte vacuoles contain vanadium and sulfate-rich homogeneous material which is easily lost during normal EM fixing procedures.[39] Kalk's paper[38] does not concern vanadocytes at all, but rather vacuolated amebocytes of *A. pygmaea*, which make up only a small percentage of differential cell counts and are believed to be an intermediate[40] in the transition from hyaline amebocytes to signet ring cells. Pirie and Bell[24] also describe a sulfate-rich cell comprising 74% of the differential cell count in blood from *Ascidiella aspersa*. Despite containing no vanadium or other hydrolysable transition metals, these cells were found to yield sufficient acid on lysis to indicate an intracellular pH of about 1. Pirie and Bell conclude:[24] "...it is, therefore, hard to avoid the conclusion that these cells contained sulfuric acid".
(15) Henze, M. *Hoppe Seyler's Z. Physiol. Chem.* **1911**, *72*, 494.
(16) The small increase in vanadyl ion concentration on formation of the Henze solution as reported in ref 1 could not represent the residue following wholesale oxidation of V(III) to vanadate since the acid produced by complete oxidation of V(III) to V(V) (~0.2 N) would have resulted in $-7/2_{\parallel}$ EPR lines of ca 11–12 G, i.e. much narrower than the 18 G observed. Furthermore, XAS examination of the Henze solution similarly prepared indicated that >90% of the vanadium was present as V(III), even in the blue, air-exposed hemolysate.[2] In all cases the cell samples discussed here were either minimally or never exposed to atmosphere. These considerations, along with the similarity in pH between the epr, and the anaerobically lysed, Henze solution samples strongly suggests that the acid was produced by a mechanism other than oxidation of V(III).
(17) Bruening, R. C.; Oltz, E. M.; Furukawa, J.; Nakanishi, K. *J. Am. Chem. Soc.* **1985**, *107*, 5298.

keeping with our previous findings.[1]

This is in contrast to the EPR results of Hawkins et al.[7] who observed only a weak signal from a vanadyl ion of unspecified composition in fresh whole blood cells from *A. ceratodes*. In their hands only exposure of the blood cells to dioxygen was said to yield material returning a strong vanadyl ion EPR spectrum. The oxidized cell spectra were found to be inconsistent with the pentaaquo ion however.[18]

In order to more rigorously test whether the vanadyl ion EPR signal we observed from intact cell preparations arose from adventitious oxidation,[19] samples of whole blood were subjected to rapid freezing immediately upon removal from individual healthy *A. ceratodes* specimens. Three independent samples were so treated, and the EPR spectrum of one of these is shown in Figure 1b. The spectra of the other two were comparable. Again a strong and easily observed vanadyl ion signal was obtained. The magnetism ($A_\parallel = 1.833 \times 10^{-2}$ cm^{-1}, $A_\perp = 0.7188 \times 10^{-2}$ cm^{-1}, $g_\parallel = 1.933$, $g_\perp = 1.992$) was again completely consistent with the pentaaquo ion. Since no opportunity was given for adventitious oxidation, the detected aquovanadyl ion must have been present in vivo. From the 28 G line width of the $-7/2_\parallel$ resonance, an intravacuolar pH of 1.8 is found, a value identical with that previously determined.[1]

In contrast to the foregoing, Hawkins et al. used whole body or body-section EPR spectra from specimens of Phlebobranch species to show that the pentaaquovanadyl ion is not contained in these organisms.[7] Indeed the spectra there displayed were not consistent with the presence of this ion. To investigate this finding, whole *A. ceratodes* specimens were flash frozen in, and granulated under, freezing pentane. Any vanadyl ion EPR signal originating from this material should be representative of the unoxidized tissue environment. The spectrum resulting from this EPR experiment is shown in Figure 1c, and is very similar to that reported to arise from unoxidized whole body material from *Phallusia julinea*.[7,20]

Comparison of the spectrum in Figure 1c with that in Figure 1b shows that the tissue sample spectrum appears characterized by a smaller $A_\parallel$ than that found for pentaaquovanadyl ion. This implys that tissue vanadyl ion senses a ligand field stronger than that provided by water.[21] The poorly defined line shape throughout the spectrum and the doubled feature near 0.325 T also imply that tissue vanadyl ion resides in more than one ligand environment.

From the discussion and data above, it is clear that the ligation sphere of vanadyl ion in ascidian tissue is not comparable with that of vanadyl ion in ascidian blood cells.[22] Therefore the conclusion[7] that whole body EPR spectra are not consistent with pentaaquovanadyl ion is correct but nevertheless has no bearing whatever on whether or not this ion is present in vanadium-containing blood cells.

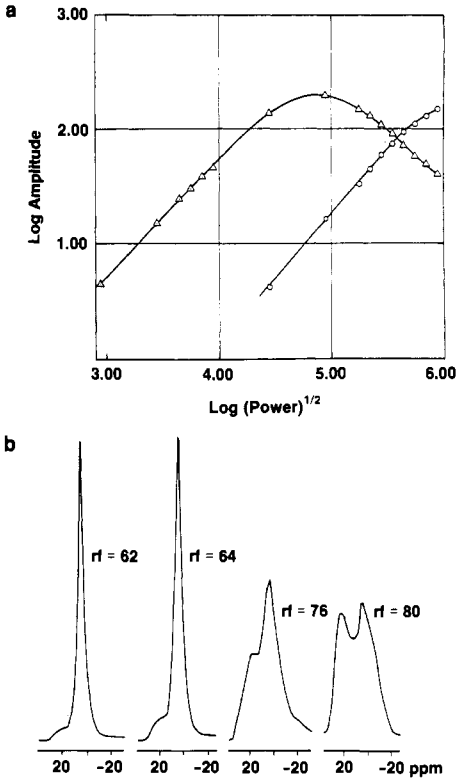

Figure 2. Progressive continuous wave (CW) NMR saturation studies on the ^{1}H NMR spectrum of intact *A. ceratodes* blood cells packed in a 5-mm NMR tube.[3] (a) Saturation curves measured at 100 MHz on a Varian XL 100 spectrometer. Amplitude is given in normalized arbitrary units and radio-frequency (rf) power is given in units of P_0, the minimum rf power of the spectrometer. Saturation data for the +4.5 ppm water signal are plotted as triangles and those of the +21.5 ppm signal as circles. (b) Proton spectra showing selective saturation of the +4.5 ppm water resonance relative to the +21.5 ppm vanadophore water resonance, as measured at 60 MHz on a wide-line NMR spectrometer (Varian DP-60). Spectrometer rf power settings are shown with each spectrum. Applied magnetic field, H, increases from left to right in each spectrum.

Proton NMR (^{1}H NMR) Studies. Vanadium(III), present in high concentration within blood cells from *A. ceratodes*,[2] possesses excellent ^{1}H NMR contact shift properties (extremely short electron-spin relaxation times and large hyperfine coupling constants). Thus the proton chemical shifts of such complexes often occur many ppm removed from the usual range of proton NMR spectra, without appreciable signal broadening.[25]

In ^{1}H NMR spectra of intact blood cell samples from *A. ceratodes*, a paramagnetically shifted signal at +21.5 ppm[26] has

(18) The magnetism of the oxidized cells was reported by Hawkins et al. as $A_\parallel = 195.0$ G (1.767×10^{-2} cm^{-1}), $A_\perp = 65.0$ G (0.602×10^{-2} cm^{-1}), $g_\parallel = 1.941$, $g_\perp = 1.894$. The magnetism of vanadyl ion within the *A. ceratodes* blood cell pack, as given in ref 1, was misreported however. The correct values are $A_\parallel = 200.8$ G and $A_\perp = 77.3$ G.

(19) Since the plasma environment in vivo is aerobic, it is not clear that casual exposure to air will in any case cause significant oxidation of intravacuolar vanadium(III). Cells from *Ascidia mentula*, whether handled in air or under nitrogen, gave equivalent EPR spectra (Bell, M. V.; Pirie, B. J. S.; McPhail, D. B.; Goodman, B. A.; Falk-Petersen, I.-B.; Sargent, J. R. *J. Mar. Biol. Assoc. U.K.* **1982**, *62*, 709). XAS examination of *A. ceratodes* blood cells routinely handled in air[2] showed less than 1% of the vanadium present to be vanadyl ion. In addition, the two further individual rapid-freeze samples of *A. ceratodes* whole blood (see text) allowed to stand for 5 and 30 min, respectively, within the syringes used in acquisition, gave slightly *less* intense EPR spectra than the sample frozen immediately.

(20) Note 12 in ref 7 remarks upon the oxidation of ascidian tissue due to condensation of liquid oxygen into the liquid nitrogen within which the samples were stored. This is indeed remarkable since not even protons are easily mobile in ice at these temperatures (Chance, B.; Pennie, W.; Carman, M.; Legallais, V.; Powers, L. *Anal. Biochem.* **1982**, *124*, 248). We have stored samples of ascidian blood within EPR tubes for more than 2 weeks, and the semireduced form of the iron–molybdenum cofactor from *Azotobacter vinlandii* nitrogenase ($E_0' = -300$ mV) for months, under liquid nitrogen with no detectable oxidation.

(21) Chasteen, N. D. *Biological Magnetic Resonance*; Berliner, L., Reuben, J., Eds.; Plenum: New York, 1981; Vol. 3.

(22) Ambient-temperature EPR spectra have been reported for whole blood preparations of *Ascidia nigra*[23] ($A_0 = 1.013 \times 10^{-2}$ cm^{-1}, $g_0 = 1.942$), and for *A. mentula*,[24] which showed a superposition of spectra from two endogenous vanadyl complexes ($A_0 = 0.9877 \times 10^{-2}$ cm^{-1}, $g_0 = 1.968$ and $A_0 = 1.06 \times 10^{-2}$ cm^{-1}, $g_0 = 1.972$; the latter values calculated here from the published spectrum), in addition to those for *A. ceratodes* shown here and in ref. 1. The data for *A. nigra* and the former parameters for *A. mentula* imply vanadyl ion within a weak ligand field such as supplied by carboxylate.[6,24] The second set of *A. mentula* parameters indicates the presence of pentaaquovanadyl ion, which constituted 75% of the V(IV) present.[24] Thus all the extant blood cell EPR parameters are similar in suggesting an aquo- or weak-field complex of vanadyl ion and are very different from the tissue sample spectra reported here and in ref 7.

(23) Dingly, A. L.; Kustin, K.; Macara, I. G.; McLeod, G. C. *Biochim. Biophys. Acta* **1981**, *649*, 493.

(24) Pirie, B. J. S.; Bell, M. V. *J. Exp. Mar. Biol. Ecol.* **1984**, *74*, 187.

(25) Swift, T. J. In *NMR of Paramagnetic Molecules: Principles and Applications*; Lamar, G. N., Horrocks, W. D., Holm, R. H., Eds.; Academic: New York, London, **1973** pp 55–83.

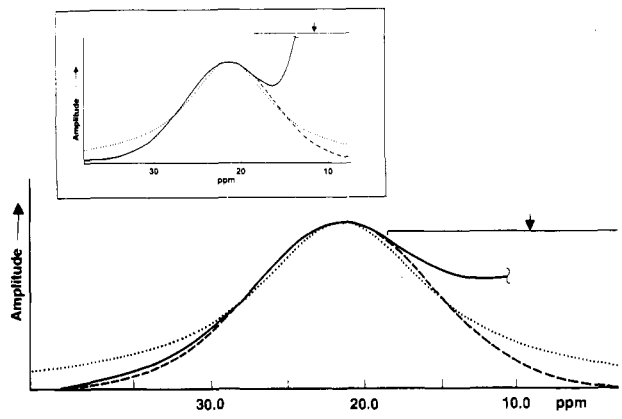

Figure 3. Curve fitting of the +21.5 ppm peak in the ^{1}H NMR spectrum of *A. ceratodes* intact blood cell preparations (cf. Figure 2 legend). The peak width at half-height measured at 100 MHz is 1380 Hz. The observed spectrum is plotted as the solid line. Both Lorentzian (dotted line) and Gaussian (dashed line) peaks of identical chemical shift, amplitude, and width at half-height were calculated and plotted. The spectrum was run on a Varian XL100 spectrometer at an rf power of 113 db where the +4.5 ppm water signal was significantly saturated, but the +21.5 ppm resonance was just at the onset of saturation (see Figure 2a). The arrow and bracket indicate the plasma water signal (+4.5 ppm) overlap region. Inset: ^{1}H NMR data of *A. ceratodes* intact blood cells obtained from Prof. J. H. Swinehart[28] using a Nicolet 200-MHz FT NMR spectrometer [one pulse sequence, 4000 scans at 1024 data points, 0.5 μs pulse width, 71 μs delay time (±7042.25 Hz spectral width), 36.42 ms accumulation time (D2 = AT, DS = 100.00 ms), 23 °C], and fit here with Gaussian and Lorentzian curves as specified above. The signal has a full width at half-height of 2370 Hz, supporting the notion (see text) that the Gaussian signal shape reflects a normal distribution of [V(III)] within the cells. The intensity of this peak is only about half that of the signal obtained by CW NMR[3], probably for reasons outlined in the text. Applied magnetic field, *H*, increases from left to right in each spectrum.

been seen along with a more intense water signal at +4.5 ppm.[3] The +21.5 ppm peak has been shown to have an intensity *directly proportional* to the number of packed blood cells in the NMR sample tube. This signal has also been observed in spectra of unpacked whole blood suspensions and been shown to disappear upon lysis of the cell sample.[3]

Continuous-wave (CW) NMR progressive saturation studies, presented in Figure 2a, show that the +21.5 ppm peak follows the saturation behavior expected[27] for a resonance with a very short spin–lattice relaxation time (T_1). Moreover, because the T_1 of the +21.5 ppm signal is significantly shorter than that of the +4.5 ppm signal, selective saturation of the +4.5 ppm peak in CW NMR spectral studies allows relative enhancement of the +21.5 ppm peak. The results of this experiment are shown in the wide-line NMR spectra in Figure 2b and by 100-MHz spectra in a previous report.[3] Curve fitting of the +21.5 ppm peak, shown in Figure 3, reveals this resonance to be almost perfectly Gaussian in shape.

The +21.5 ppm Gaussian signal, too intense to represent protons within a stable V(III) complex, was concluded to arise from the intravacuolar water in rapid exchange with the liganding waters of a labile aquovanadium(III) complex, contained within the same vacuole. The Gaussian shape of the signal was suggested[3] to represent a normal distribution of Lorentzian signals arising from a normal distribution of V(III) concentrations among the vanadium-containing vacuoles. These conclusions were supported by further experimental studies.[2,3,5,6] Subsequently, Kustin and coworkers[4] reported a similar pmr spectrum (an apparently Gaussian peak at ca. +20 ppm) to derive from a packed intact blood cell sample from *A. nigra*.

However, Hawkins et al.[7] reported that they could not reproduce the +21.5 ppm ^{1}H NMR signal on examining packed cells from *A. ceratodes*. They have further asserted that the low-field signal reported by ourselves and others is actually an artifact arising from improper phasing of the spectrometer. To support this assertion, a poorly phased proton spectrum of *A. ceratodes* packed blood cells, recorded on a 200-MHz FT NMR spectrometer, was presented.

This spectrum was discussed with reference to unpublished ^{1}H NMR spectral data of *A. ceratodes* packed blood cells, obtained from Prof. J. H. Swinehart at UC Davis, which were acquired by using *the same* 200-MHz spectrometer. In their discussion, Hawkins and co-workers directly implied that the unpublished data of Swinehart (which were not shown) are equivalent to their own, with the exception that the 21 ppm peak, which occurred to lower field than the +4.5 ppm peak in the data from Swinehart, now occurred to higher field in their own spectrum. This distinction in 21.5 ppm peak position was ascribed purely to the direction of the phasing error in the +4.5 ppm signal.

We therefore present here a more detailed comparative analysis of the unpublished ^{1}H NMR data obtained from Prof. Swinehart[28] on blood cell packs from *A. ceratodes* using FT NMR, with the spectra obtained with CW NMR.[3] In addition, assessment of the experimental data of Hawkins et al. is made, especially in light of their use of an FT NMR instrument to obtain broad-line NMR data.

Close inspection and comparison of the FT NMR data from Swinehart with that in ref 7 reveals several obvious and important differences:

1. In a comparison of ^{1}H NMR spectra with the 4.5 ppm water resonance plotted on scale, the data from Swinehart show a clearly visible peak at +21.5 ppm, whereas the published spectrum of Hawkins et al.[7] shows no sign of any resonance either to high or low field, other than the +4.5 ppm peak.

2. Curve fitting of the +21.5 ppm peak in the data from Swinehart, obtained at higher gain, shows it to be *precisely* Gaussian in shape, as shown in the inset to Figure 3. No curve fitting of the data presented in ref 7 was possible for reasons discussed below.

3. In the spectra from Swinehart, the half-height width of the +4.5 ppm peak is nearly twice that in the data obtained by Hawkins et al. (see below).

4. The +21.5 ppm peak in the data obtained from Swinehart returns to the base line near +35 ppm (Figures 3 and 4) and 80% to the base line by +30 ppm. However the −22 ppm feature in ref 7, has only returned ca. 13% to the base line by −30 ppm at which point the displayed spectrum was truncated (see Figure 4). It is impossible to calculate a fit to any spectral feature under these conditions. However if it is assumed that this −22 ppm deflection is indeed a severely truncated Gaussian peak, then it must have a peak width at half-height of 35 ppm. This is nearly *three times* larger than that found for the +21.5 ppm signal observed by Swinehart (Figure 3 caption) on the same spectrometer.

We know of no way a phasing error could give rise to the purely Gaussian +21.5 ppm peak, observable in both CW and FT ^{1}H NMR spectra of intact *A. ceratodes* blood cells and exhibiting the rf saturation behavior shown in Figure 2 as well as having all the other properties discussed above and elsewhere.[3] Furthermore, while clear Gaussian peaks are present at +21.5 ppm from TMS (+17 ppm from water) in both the data from Swinehart and of ourselves, only a sloping, misphased base line is apparent in the spectrum of Hawkins et al.,[29] as shown in Figure 4. We therefore believe that a more likely interpretation of the −22 ppm feature reported in ref 7 is that it is the slowly descending

(26) In order to maintain consistent convention with the notation in ref 7, positive values of ppm refer to a lower field than TMS and negative values of ppm to higher field.

(27) Poole, C. P. and Farach, H. A. *Relaxation in Magnetic Resonance*; Academic: New York, London, 1971.

(28) We are very grateful to Prof. J. H. Swinehart (Department of Chemistry, University of California, Davis, CA 95616) for supplying us with copies of his unpublished spectra and for permission to show them herein.

(29) The −22 ppm feature has in any case the wrong chemical shift to support the symmetrical phasing error argument advanced by Hawkins et al.[7] A symmetrical phasing anomaly should have placed the companion to the +21.5 ppm peak at −12.5 ppm rather than at −22 ppm because the large water resonance, which Hawkins et al. suggest gave rise to their −22 ppm feature by incorrect phasing, occurs at +4.5 ppm.

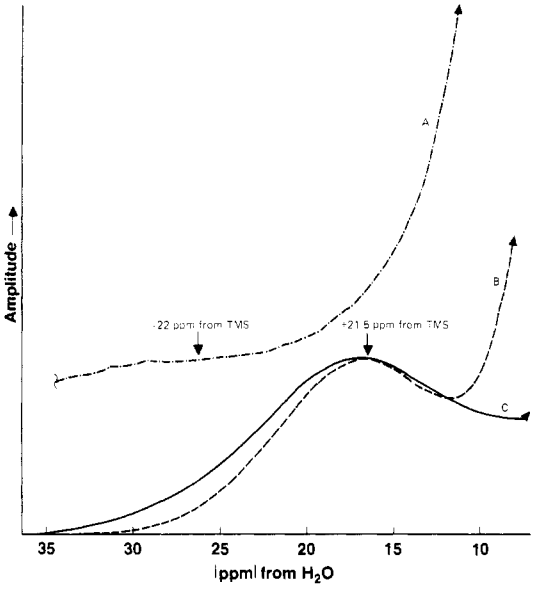

Figure 4. Comparison of normalized ^1H NMR data for *A. ceratodes* packed intact blood cells: (A) data of Hawkins et al[7] obtained on a Nicolet 200-MHz FT NMR spectrometer; (B) data from Swinehart[28] obtained on the same Nicolet 200-MHz FT NMR spectrometer as used by Hawkins et al. in acquiring the data in A; (C) data obtained previously by using a 100-MHz CW NMR spectrometer. Spectra are plotted in absolute value of ppm from water (+4.5 ppm from TMS) in each spectrum. The data in part A are from the high-field side of the water signal while those in parts B and C are from the low-field side (see text). Amplitude data were normalized to the maximum peak height of the +21.5 ppm (from TMS) signal (+17 ppm from water) for parts B and C but to −22 ppm from TMS (−26.5 ppm from water) for part A to examine the assertion[7] that this signal arose from a symmetrical phasing anomaly centered about the +4.5 ppm water signal.[29] Spectrum B rises more steeply to higher field than spectrum C because the latter spectrum was run under conditions that partially saturated the +4.5 ppm water peak (see Figures 2 and 3).

base line associated with a poorly phased spectrum or alternatively an anomaly associated with the use of inappropriate FT NMR data acquisition parameters as discussed below. However, since the spectrum reported in ref 7 was so severely truncated, whether the line shape traces out a broad peak beyond −30 ppm or simply is a slowly descending base line can not be determined.

The question of why Hawkins et al. did not see the +21.5 ppm Gaussian peak, which is easily observable in CW ^1H NMR spectra of intact *A. ceratodes* blood cell packs, remains. Resonances having extremely short relaxation times (T_1 and T_2) and unusually large chemical shift ranges can be difficult to study by using the typical high-resolution FT NMR procedures. For example, texts[30] on FT NMR caution that broad (short T_2) signals are suppressed relative to narrower signals when too long a delay time is used in data acquisition. The T_2^* associated with the +21.5 ppm signal is over 10^3 times shorter than that of peaks usually observed in high-resolution FT proton NMR. In addition to suppressing[31] the broad +21.5 ppm resonance, an inappropriately long delay time would tend to cause a narrowing[32] of the remaining inhomogeneously broadened[33] +4.5 ppm resonance in packed cell spectra. The spectrum shown in ref 7 is in fact only about half the width of that from Swinehart[28] as noted above.

Other instrumental precautions and pitfalls associated with attempts to study broad lines with unusually large chemical shift ranges by using high-resolution FT NMR spectrometers could be discussed.[30] However since ref 7 provides none of the FT NMR instrumental parameters used in accumulation or processing of the displayed data it is not possible to further assess this matter.

From the data and discussion presented above, therefore, the most reasonable interpretation of the XAS, NMR and EPR data currently available regarding the inorganic chemistry of the blood of *A. ceratodes* is as follows:

1. XAS experiments[2] show that the intravacuolar vanadium is trivalent, exists free of any rigid, stable chelate, and is enclosed in a ligand array of nearest neighbor low-Z donor atoms (nitrogen or oxygen).

2. ^1H NMR studies[3] yield good evidence that about four of these ligands derive from water. Since V(III) hydrolyzes extensively above pH 2.5, the two results taken together imply an acidic vacuolar pH.

3. EPR spectra of intact blood cells[1] show that endogenous pentaaquovanadyl ion senses a pH of about 1.8. Although application of this value to vanadium containing vacuoles assumes the cocompartmentalization of VO^{2+} and V(III), this assumption is reasonable. A low-to-moderate intravacuolar viscosity is also assumed. The derived pH is however, completely consistent with the requirements of points 1 and 2 above.

4. Sulfur X-ray absorption near-edge structure (XANES) spectra[5,6] of whole blood cell packs have shown large amounts of intracellular sulfate, supporting the notion that the endogenous V(III) is principally maintained as a sulfato complex ion.[1,34] This conclusion is reinforced by the observed broadening of the sulfate XAS edge feature in both the cellular spectra and in spectra of vanadium(III) sulfate solutions. This effect appears to characterize sulfate-complexing cations.[35]

To conclude this discussion we emphasize that our results are strictly applicable only to *A. ceratodes*. Specific extrapolations to other species of tunicate should be made tentatively, particularly in view of the emerging understanding of variations in blood chemistry among even closely related organisms.[36]

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Registry No. V, 7440-62-2; $VO(H_2O)_5^{2+}$, 15391-95-4; VO^{3+}, 12192-26-6.

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(36) For example, in *Ascidia mentula*[24] and *A. callosa Stimpson*[37a] the cellular distribution of vanadium content is morula cells > signet ring cells > compartment cells, but is amebocytes > signet ring cells > compartment cells with only traces of V in morula cells from *Ciona intestinalis* and *Phallusia mamillata*.[37b,c] In *Ascidia nigra*, V is apparently found localized in signet ring cells with only traces in morula cells.[37d] Therefore significant differences can apparently occur even at the species level.
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