Nuclear Magnetic Resonance Spectrum of Living Tunicate Blood Cells and the Structure of the Native Vanadium Chromogen

[contact shift/Ascidiav vanadium(III)/hemovanadin/vanadocyte]

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ABSTRACT The $^1$H nuclear magnetic resonance spectrum of living tunicate blood cells was examined in an attempt to develop a biophysical assay for the native vanadium chromogen. The living cell spectrum was found to exhibit a broad 21 ppm downfield Gaussian signal which, however, disappears immediately upon cell disruption. Examination of the properties of this extremely low field signal revealed that it corresponds to a labile vanadium(III) aquo complex contained in the cell vacuoles, that vanadium(III) concentrations are rigidly regulated within these vacuoles, and that artifact formation does occur in the hemolysate. The living cell spectrum also indicates the number of ligand-bound vanadium(III) coordination sites in the native blood pigment. Results are discussed in relation to the possible functions of the vanadium chromogen.

The presence of a vanadium chromogen in the blood of certain tunicates (a group of strictly marine filter-feeding animals related to the vertebrates) is one of the most fascinating and puzzling marine biochemical phenomena. The basic structure and function of other blood metallochromogens (hemoglobin, chlorocruorin, hemerythrin, and hemocyanin) have been established, and although substantial effort has been expended in the study of the vanadium chromogen (hemovanadin), its structure and function remain controversial. More than 50 articles, including several reviews, concerning hemovanadin have appeared since Henze discovered the chromogen over 60 years ago (1-6).

The uncertainty concerning the chemical structure of the vanadium chromogen arises from its instability. The extraordinarily unstable green chromogen is enclosed in a 2-$\mu$m diameter vacuole (vanadophore) housed in an 8-$\mu$m diameter blood cell (vanadocyte) (7). Past studies indicate that the chromogen contains vanadium almost exclusively in the air-oxidizable, tripositive oxidation state and that the chromogen is maintained in a strongly acidic sulfuric acid solution which is contained in the vanadophore (5, 6). Although living vanadocytes possess a light green color, immediately upon lysis a dark red-brown solution is formed (Henze solution) (5). In Henze solution, vanadium(III) has been found associated with a protein and/or a small nitrogenous molecule of unknown structure (5, 6). It seems certain that the vanadophore encloses the vanadium chromogen-sulfuric acid solution to protect the cell from the vacuole's strongly acidic and reducing conditions and to maintain proper conditions in the vanadophore for the function of the chromogen. Opening the vanadophore in the presence of extravacuolar cytoplasm clearly constitutes a highly unphysiological condition and offers opportunity for denaturation and artifact formation in the hemolysate. The problem is further complicated by the fact that although tunicate blood contains numerous cell types, only one contains the green vanadium chromogen (2, 3, 7). No criteria for determining the absence of artifacts in material isolated from the hemolysate have been devised.

Since past studies may have dealt with artifacts having no physiological significance, some method of assay for the physiologically active vanadium chromogen was needed. Development of a functional biochemical assay for the native vanadium chromogen would be extremely difficult because neither the biological function of the vanadocyte nor the biochemical function of the vanadium chromogen has been established. Since there is good evidence that the native chromogen is a vanadium(III) complex and since vanadium(III) complexes possess excellent nuclear magnetic resonance (NMR) contact shift properties (extremely short electron-spin relaxation times and large hyperfine coupling constants), the possible development of a biophysical assay for the physiologically active vanadium complex was explored. The NMR signals from protons of ligands bound to vanadium(III) ions can be shifted many Hertz up or downfield without appreciable signal broadening (8-10). The NMR spectrum of living vanadocytes, therefore, could exhibit paramagnetically shifted signals due to a vanadium(III) complex if the signals were narrow enough to be observed and if the signal shifts were great enough to allow their resolution from the massive signal due to intra- and extracellular water. The position and intensity of the paramagnetically shifted signals could then be used as an assay for the isolation and structural determination of the organic ligand associated with vanadium(III) in vivo. The proton NMR spectrum of living vanadocytes was found to exhibit a paramagnetically shifted signal which, however, disappeared immediately upon cell lysis. The following is a report on the examination and interpretation of the proton NMR spectrum of living vanadocytes.

Living vanadocyte NMR spectrum

Healthy, uninjured specimens of the tunicate Ascidiacia ceratodes (Huntsman, 1912) were collected from either Monterey Bay or Tomales Bay, Calif. (locations 300 km apart) during all seasons over a 2-year period. The tubular heart was exposed, punctured, and cannulated near its anterior end and blood was withdrawn at a rate sufficiently slow to prevent collapse of the heart and damage to the blood cells. The proton NMR spectrum of eight packed cell preparations was taken over the 2-year period. The NMR spectrum of each packed cell preparation exhibited a broad signal approximately 21 ppm downfield from sodium 2,2-dimethyl-2-silapentane-5-sulfonate and a massive water signal (Fig. 1b). The mean value for the

Abbreviation: NMR, nuclear magnetic resonance.
position of the low field peak for the eight samples was found to be 21.5 ppm, with a probable error of the mean of 0.1 ppm. The signal width at half-height was 1300–1400 Hz (measured at 100 MHz). Line shape analysis showed that the 21 ppm peak was Gaussian, whereas the 4.5 ppm peak was Lorentzian.

To be certain that the 21 ppm peak was not an artifact of cell packing, a spectrum of unpacked whole blood was run. The spectrum of a sample of 2.22 ml of fresh blood was determined immediately after withdrawal in a 12 mm diameter tube using the XL-100 NMR spectrometer. The spectrum exhibited a peak 21.7 ppm downfield from water, demonstrating the peak’s presence in the spectrum of living unpacked cells (Fig 1a). A packed cell sample containing 4.8 times more cells than the unpacked cell sample had a 21 ppm signal intensity 4.8 times greater, demonstrating that the intensity of the 21 ppm signal is directly proportional to the number of cells present.

The low field signal is observed in spectra run at both the normal physiological temperature (14°C) for *A. ceratodes* and the normal spectrometer operating temperature (33°C). At the normal spectrometer operating temperature, the vanadocytes in the NMR tube eventually hemolysed, as indicated by the contents of the NMR tube turning dark red-brown and by a drastic drop in plasma pH. Repetitive spectra of a packed cell preparation that had been in the spectrometer for an extended period of time revealed an interesting phenomenon: concurrent with lysis of the vanadophores, the 21 ppm peak shifts upfield and decreases in amplitude as it moves (Fig 2). The process is complete within 12 min, with the 21 ppm signal disappearing into the water signal. The 21 ppm peak is absent in the spectrum of the hemolysate. These data can be summarized as follows: (i) A 21 ppm signal can be observed for any sample of healthy *A. ceratodes*’ blood, and the intensity of this signal is directly proportional to the number of blood cells present. (ii) The 21 ppm peak is quite broad, 1300–1400 Hz wide, and is Gaussian in shape. (iii) The 21 ppm signal can be observed only when intact vanadocytes are used. (iv) As the vanadophores lyse, the 21 ppm signal shifts upfield with a concurrent decrease in amplitude.

These data suggest that the 21 ppm signal in the spectrum of intact vanadocytes is due to vanadophore solvent water protons in rapid exchange with the protons of water in the coordination sphere of vanadium(III) inside vanadophores. Since the 21 ppm signal is extremely broad yet of measurable amplitude, it must be due to a large number of protons in the environment characterized by the signal, and it is impossible with the quantity of vanadium present that protons of a stable vanadium(III) complex could account for so massive a signal. At 33°C the water protons in the coordination sphere of vanadium(III) and bulk solvent water protons are in rapid exchange, and only a single weight averaged signal is observed (8, 11). Under these conditions the bulk water signal is shifted downfield by the contact interaction of the water protons with the unpaired electrons of the paramagnetic vanadium(III) ion. The solution water shift, $\Delta \omega$, relative to pure water for this rapid exchange limit, is directly proportional to the concentration of water molecules in the first coordination sphere of vanadium(III), provided vanadium(III) is the only paramagnetic ion present, and provided the vanadium(III) concentration is low compared to water. $\Delta \omega$ is given by Eq. [1]:

$$\Delta \omega = q p \Delta \omega_M$$  

where $\Delta \omega_M$ is the resonance frequency of water protons in the first coordination sphere of the vanadium(III) ions relative to pure water, $q$ is the number of coordination sites free to interact with solvent water molecules [maximum of six for the hexaqua vanadium(III) ion], and $p$ is the vanadium(III) molal concentration (12–14).

As the vanadophores lyse, the vacuolar contents mix with extravacuolar cytoplasm. This results in dilution of vanadophore preparations undergoing time-dependent lysis. (a) 0 min, vanadophores are intact and are light green in color; (b) 4 min, vanadophores are beginning to lyse and the 21 ppm NMR resonance shifts toward the water resonance and decreases in intensity; (c) 8 min, lysis continuing with concomitant NMR shifts; (d) 12 min, cell lysis complete and the lysed vanadophore solution is dark red-brown and the 21 ppm NMR resonance is absent.

**Fig. 2.** NMR spectrum (measured at 60 MHz) of vanadocyte preparations undergoing time-dependent lysis. (a) 0 min, vanadophores are intact and are light green in color; (b) 4 min, vanadophores are beginning to lyse and the 21 ppm NMR resonance shifts toward the water resonance and decreases in intensity; (c) 8 min, lysis continuing with concomitant NMR shifts; (d) 12 min, cell lysis complete and the lysed vanadophore solution is dark red-brown and the 21 ppm NMR resonance is absent.
dium(III), lowering $p$ in Eq. [1], some oxidation vanadium(III) to vanadium(IV), also lowering $p$, and possible combination of vanadium(III) with extravacuolar cytoplasmic constituents, lowering $q$ in Eq. [1]. Each of these processes would result in an upfield shifting of the 21 ppm signal, as is observed in the spectrum of packed cell preparations upon hemolysis (Fig. 2).

Since the 21 ppm peak is approximately Gaussian in shape, it possibly represents a distribution of vanadium(III) concentrations in the vanadophores. Lysis of the vanadophores would result in a collapse of the vanadium(III) concentration distribution into a single concentration, resulting in a significant narrowing of the 21 ppm peak. Dilution of vanadium(III) during vanadophage lysis would result in a smaller ratio of coordination sphere protons to solvent protons, while the total number of protons undergoing exchange would increase. Dilution, therefore, would produce a decrease in signal width and an increase in total intensity of the 21 ppm signal as it shifted upfield. Oxidation of vanadium(III) released by hemolysis, however, would result in a broadening of the 21 ppm signal, since vanadium(IV) greatly broadens water signals (enhances relaxation) but does not cause appreciable paramagnetic signal shifts (15). In addition, formation of high-molecular-weight vanadium(III) artifacts would also enhance solvent proton relaxation (16). The fact that the 21 ppm signal decreases in amplitude as it shifts upfield indicates oxidation and/or high-molecular-weight artifact formation rather than a simple dilution effect as vanadophores lyse.

Microscopic measurements compared with signal intensity

If the 21 ppm signal is attributable to water protons in rapid exchange with vanadium(III) enclosed in the vanadophores, then the intensity of the 21 ppm signal should be a measure of the amount of water in the vanadophores in the NMR tube. This proposition can be tested by comparing microscopic measurements of the volume of vanadophores with NMR intensity data. Microscopic measurements and NMR measurements were made with specimens of A. ceratoles taken at the same time and location at Monterey Bay. The microscopic measurements are described and the results of the measurements are presented in the legend to Fig. 3. The NMR packed cell sample was prepared from 10.7 ml of blood. From the data presented in Fig. 3, the volume of the vanadophores in the NMR tube amounted to 0.028 ± 0.008 ml.

NMR spectral intensities were used to determine the quantity of water corresponding to the intensity of the 21 ppm peak. The volume of the NMR packed cell sample was 0.47 ml. The intensity of the 21 ppm peak in the spectrum of the 0.47-ml cell sample was correlated with the total spectral intensities of several 0.47-ml electrolyte samples of known water content. Saraf and Fatt (17), using a single coil NMR spectrometer, reported that the NMR signal intensity of water in electrolyte solutions can vary with solution conductivity, independent of proton content. When their experiments were duplicated with a cross coil NMR spectrometer (Varian XL-100), it was found that for samples of equal water content, the measured water signal intensities were equal to within 3% over the entire conductivity range. Since the 21 ppm peak was Gaussian in shape, its intensity equals $\frac{(\pi/\ln 2)^{1/2}\Lambda_0 Y_S}{Y_0}$ where $\Lambda_0$ is the peak width at half-height and $Y_0$ is the maximum peak amplitude.

![Fig. 3. Vanadocyte blood cells of A. ceratoles containing green vacuoles (vanadophores). Phase contrast photomicrograph of fresh vanadocytes, magnification ×1800. Cells (middle and right of picture) are compressed slightly under cover slip to allow accurate determination of the number of vanadophores per vanadocyte. The average number of vanadophores per vanadocyte was 14.0, with a probable error of the mean $Q$ of 0.3 for 40 measurements. The white circular area inside each vanadocyte is the cell nucleus. Vanadophage diameters were measured to within 0.3 μm, using a micrometer eyepiece and uncompressed vanadocytes containing approximately spherical vanadophores, and vanadophage volumes were calculated assuming spherical shape. The average vanadophage volume was $5.0 \times 10^{-12}$ cm$^3$ with a $Q$ of $0.3 \times 10^{-12}$ cm$^3$ for 60 measurements. Total and differential cell counts were performed by the procedure of Vallee (26). The blood contained an average of $64 \times 10^8$ cells per cm$^3$ with a $Q$ of $13 \times 10^8$ cells per cm$^3$ for 25 measurements. Vanadocytes represented an average of 37% of the total number of cell types with a Q of 1% for 10 measurements. (Photomicrograph by Dr. K. J. Judy.)](image-url)
Vanadium content and the 21 ppm signal

As demonstrated above, the 21 ppm signal corresponds to water protons in rapid exchange between the solvent and the coordination sphere of vanadium(III) in the vanadophore. Unanswered, however, is how much of the vanadium present in \textit{A. ceratodes} blood is accounted for by the 21 ppm signal. Atomic absorption vanadium analysis, volumetric sulfate analyses, and NMR vanadophore water content analysis indicate maximum vanadium(III) and sulfate molal concentrations of 1.08 molal and 1.59 molal, respectively. Since the vanadophore signal shift magnitude (21.5 ppm) is related to the molal concentration of vanadium(III) in the vanadophore, it should be possible to determine the vanadium(III) molal concentration in the vanadophore by comparison with the bulk resonance shifts of water in the spectra of suitable vanadium(III) standard solutions. Comparison of the vanadium(III) molal concentration determined from atomic absorption and NMR intensity data with NMR shift data would indicate how much vanadium is accounted for by the 21 ppm vanadophore signal, and would give the maximum number of vanadophore vanadium(III) coordination sites interacting with ligands other than water in \textit{A. ceratodes}.

Paramagnetic bulk susceptibility effects can cause significant contributions to resonance shifts measured relative to an external reference (19). The position of the vanadophore 21 ppm resonance was measured relative to the plasma water resonance, the plasma water being an external reference, i.e., not enclosed in the vanadophores. The spectrum of coaxial 5 mm and 12 mm diameter tubes containing packed blood cells and 98% deuterium oxide, respectively, showed a split water resonance (Fig. 1b). The larger plasma water signal is shifted upfield relative to the proton signal of water in 98% deuterium oxide due to the presence of paramagnetic vanadium(III) in the vanadophores of the packed cell sample. The plasma water, therefore, is not a reference external to the vanadophores with respect to bulk susceptibility effects since the plasma water resonance position is affected by the paramagnetic susceptibility of vanadium(III) contained in the vanadocytes.

Since bulk susceptibility effects depend primarily on sample and magnet geometries (20), spectra of identical packed cell preparations were determined on spectrometers of different magnet geometries (100 MHz and 300 MHz spectrometers), and the resonance position of the vanadophore water signal was found to be 21.5 ppm for both. The bulk susceptibility correction that must be applied to the vanadophore water resonance when measured relative to the plasma water resonance, therefore, is negligible because, although different bulk susceptibility shifts are expected for spectrometers of different magnet geometries, no shift difference was observed; the plasma water, therefore, functions as an internal reference.

Standard curves of water resonance shifts against vanadium(III) concentrations in perchloric acid and sulfuric acid solutions were prepared (Fig. 4). The shifts for the vanadium(III) solutions were measured relative to an internal deionized water reference and referred to pure water. The difference in slope in Fig. 4 for vanadium(III) water resonances in sulfate and perchlorate media indicates complexation of sulfate (14). The molal concentration of vanadium(III) corresponding to the 21 ppm vanadophore signal can be evaluated from Fig. 4.

Several important assumptions were made in evaluating the 21 ppm signal in terms of Fig. 4: (i) The solvent water protons are in rapid exchange with the water protons in the first coordination sphere of vanadium(III) ions enclosed in the vanadophores. Vanadophore water resonance shifts measured above 20°C follow the Curie law predicted for the fast exchange conditions. (ii) Vanadium(III) is the only paramagnetic ion that occurs at a significant concentration in the vanadophore. Iron has been found to be the only paramagnetic metal ion other than vanadium that occurs in significant quantities in \textit{A. ceratodes} blood (21). Atomic absorption iron and vanadium analyses of three packed cell digests of \textit{A. ceratodes} prepared over a 6-month period, however, revealed an iron concentration less than 2% of the vanadium concentration for all three samples. There is no evidence that the iron is contained in vanadophores. (iii) \( \Delta \omega_M \) of the vanadophore vanadium(III) aquo-complex equals \( \Delta \omega_M \) of the vanadium(III) sulfuric acid aquo-complex used in making the measurements presented in Fig. 4. Similar assumptions concerning variations in \( \Delta \omega_M \) for other transition metal complexes have been made and discussed in past studies (14). \( \Delta \omega_M \) has been directly measured for the hexaaquovanadium(III) complex (11) and can, in principle, be measured for the vanadium(III) aquo-complexes of both vanadium(III) sulfate solutions and vanadophores. Preliminary low-temperature (210 K), wide-line NMR studies of frozen vanadocytes (unpublished data) support this assumption.

The 21.5 ppm vanadophore water resonance is 17.1 ppm downfield from the plasma water resonance and 15.5 ppm downfield from the proton resonance of a 1.54 molal sulfuric
Acid solution. A 15.5 ppm shift corresponds to a 0.65 molal hexaqua-vanadium(III) ion solution or a 0.85 molal vanad-
dium(III) sulfate solution (Fig. 4). The weight of water present in the vanadophores of the NMR sample was 0.033 g, which corresponds to a total of 21 µmol of vanadium for a 0.65 molal solution and 28 µmol of vanadium for a 0.85 molal solution. The NMR sample contained a total of 35 µmol of vanadium. Twenty-one and 28 µmol of vanadium are 60% and 80% of 35 µmol, respectively. If all the vanadium in the packed cell sample was present as vanadium(III) in the vanadophores and free to bind sulfate ions (5), then 80% corresponds to five of the six possible vanadium(III) coordi-
nation sites, i.e., a maximum of one coordination site per vanadium(III) ion would be left available for binding an organic ligand. Occurrence of unreduced vanadium in possible vanadocyte stem cells, demonstrated by electron micro-
scopic studies (18), would further reduce the number of available vanadium(III) coordination sites. The picture of limited complexation of vanadium(III) in vanadophores agrees with the hypothesis developed by Bielig et al. (5). Limited complexation of vanadium(III) ions enclosed in living vanadocytes does not indicate that the vanadophore represents an inactive store of vanadium because: (i) statisti-
cal analyses of the 21 ppm peak shift variation interpreted using Fig. 4 indicate that the vanadium(III) concentration is strictly regulated in the vanadophore, the mean concentra-
tion varying only 0.02 molal from animals of greatly varying origins, and (ii) 21 ppm peak widths indicate that within any single sample of blood the vanadium(III) concentration range of the vanadophores is sharply limited. Since it is im-
probable that the tunicate would regulate the concentration of a stored inactive compound so rigidly, it is reasonable to 
assume that the vanadophore is a functional unit. 

Function of the vanadium chromogen
Tunicate blood hemolysate does not oxygenate reversibly, and it has long been thought that the vanadium chromogen is not involved in oxygen transport (22). Carlisle has shown, however, that intact living tunicate blood cells can act as oxygen storage and transport units (6). The present study has demonstrated that artifact formation does occur in the hemolysate, and it is reasonable to assume, therefore, that vanadocytes possess properties that cannot be duplicated by conventional hemolysates. It has been alternatively proposed that the vanadocyte (i) transports, stores, and processes nutrients (23); (ii) is involved in the production of the poly-
saccharide outer covering of the animal (7), a theory con-
tested by labeled glucose studies (24), or (iii) is involved in the antimicrobial defense mechanism (25). These hypotheses are based primarily on histological observations and do not suggest a biochemical reason for the presence of a concen-
trated acidic vanadium(III) sulfate solution in the vanad-
ophore. It has been proposed that the vanadium chromogen functions in the production of acid for an unknown purpose (7, 21). However, the blood of certain tunicates contains highly acidic large vesicular cells that are devoid of the va-
adophore chromogen (2, 9). It is generally felt that the reduc-
ing properties of the vanadium chromogen are the key to its function (6).

This study demonstrates that the native vanadium chromo-
gen enclosed in living vanadocytes is essentially a vanadium-
(III) solution with a rigidly regulated concentration. These 
findings suggest that unlike the metalloprotein macromole-
cules of the other blood metallochromogens, it may be neces-
sary to look at the vanadophore vacuole, and perhaps the 
vanadocyte as the smallest functional unit. The findings fur-
ther suggest that conventional biochemical procedures using 
the disruption of whole blood cells by methods that result 
in significant alterations in the vanadium(III) concentration 
and that offer the opportunity for artifact formation with 
extravacuolar cytoplasm are likely to yield spurious results 
that do not reflect the true physiological function of the native 
chromogen in the intact cells.

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1. Henze, M. (1911) Hoppe-Seyler's Z. Physiol. Chem. 72, 
494–501.
5. Bielig, H. J., Bayer, E., Dell, H. D., Rohns, G., Mollinger, 
Chem. 6, 1315–1320.
Chem. 6, 1607–1613.
325–331.
12. Swift, T. J. (1973) in NMR of Paramagnetic Molecules: 
Principles and Applications, eds. Lamar, G. N., Horrocks, 
W. DeW. & Holm, R. H. (Academic Press, New York and 
307–320.
3766.
4656.
21. Swinehart, J. H., Biggs, W. R., Halko, D. J. & Schroeder, 
23. Andrew, W. (1965) Comparative Hematology (Grune and 
126, 267–280.
276–279.
6, 1948–1951.