of activation is also expected, since the reaction entails two oppositely charged ions forming a neutral product. The release of tightly solvated water molecules from the ions upon forming the activated complex is the source of increased entropy and corresponds to a decrease in electrostriction.[38] The protein provides the positively charged heme with a hydrophobic environment, which increases the effective charge on the iron. This in turn is responsible for a greater attraction between the iron and cyanide and a subsequent decrease in the activation enthalpy relative to the model system. This increased Coulombic attraction is also manifested in the extremely small reverse rate constant for MbCN. Once the charge in the low dielectric hydrophobic pocket is neutralized by addition of cyanide to iron, the complex will only dissociate slowly.

The activation entropies in enzyme substrate kinetics are thought to be influenced by solvation and structural effects.[39] In enzyme substrate reactions where the substrate is an ion and subsequently neutralized, the activation entropy is usually positive. The negative activation entropy for the MbCN reaction suggests that the conformational contribution is dominant. Large negative activation entropies are common in protein substrate kinetics.[39] The conformational effects which contribute to negative activation entropies arise from the protein changing reversibly to a closed or rigid structure in the activated complex, which has fewer vibrational and rotational modes available. In the MbCN case, steric interactions of cyanide with the distal residues would produce a crowded environment in the heme pocket. The opening to the binding site must be enlarged, to release the metal-bound water and allow cyanide to enter, which would decrease the motion of the residues at the opening. A concerted mechanism of these events occurring simultaneously is unlikely. A more plausible explanation involves multiple reaction barriers, similar to those observed for MbCO.[40] The observed activation entropy in the present case could arise from a combination of negative and positive activation entropies of individual barriers. Nevertheless, a rigid conformation in the activated complex is indicated by the large negative activation entropy for MbCN formation. The exact nature of the "activated complex" cannot be determined, but the possibilities outlined above suggest a crowded environment around the binding site.

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Conclusion

The increase in Coulombic attraction between cyanide and metmyoglobin accounts for the larger affinity constant of MbCN compared to the model system, OPCN. This effect is demonstrated in the equilibrium constant analysis for MbN_3 and OPN_3, in theoretical calculations, and in the kinetics of cyanide binding and dissociation (and the associated activation enthalpy). The large negative activation entropy for MbCN formation indicates that there is a large entropically unfavorable conformational change in forming the complex.

An estimate of the change in free energy due to steric interactions of linear ligands with myoglobin was obtained. This semiquantitative result implies that steric effects are smaller than previously thought.[9,2b] Analysis of MbCO and MPCO equilibrium data, using this estimate of the steric free-energy change, suggests the possibility of differences in the M–L bond strength due to these steric interactions.

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Structural Study of the Vanadium Complex in Living Ascidian Blood Cells by X-ray Absorption Spectroscopy

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Abstract: The immediate coordination environment of vanadium in living ascidian blood cells (vanadocytes) has been studied by X-ray absorption spectroscopy. Analysis of the X-ray absorption edge data shows clearly that only a small amount (less than 10%) of the vanadium is present as the VO^{2+} ion in either living or spontaneously lysed vanadocytes. Parameters for curve-fitting analysis of V EXAFS were determined semiempirically from data on model complexes of known structure and have been tested for amplitude and phase transferability by using other structurally characterized V complexes. Analysis of the EXAFS data from the vanadocytes establishes that the vanadium in the living cells is present as V(III) ions surrounded in the first coordination sphere by a symmetric, single shell of low-atomic-weight scatterers. The data are best fit by a single shell of six oxygen atoms at an average distance of 1.99 Å. The EXAFS data show no evidence for the presence of VO^{2+}. Absence of a well-defined second shell of scatterers suggests that the vanadium complex in the living cells is simply aquovanadium(III), not associated with a ligand containing ordered, more distant shells (such as porphyrin or imidazole). Upon spontaneous lysis, little change in the vanadium coordination environment (as judged by the edge or EXAFS) is observed.

Studies of metal ions in biological systems have been successful in defining a wide variety of biochemical processes which make use of the special properties of metals.[1] However, one such system has confounded the efforts of several generations of scientists to discover its biochemical significance. This is the vanadocyte, the vanadium-sequestering cell of tunicates[2] (widely distributed, marine filter feeding animals classified within a subphylum of the Chordata[3]). Its nature is such that neither the function of this

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unique cell, nor the structure of the vanadium complex contained within, has yet been elucidated. We report here the use of X-ray absorption spectroscopy to study the structure of the vanadium complex in living tunicate blood cells.

The vanadocyte is indeed very different from the blood cells of other animals.[4] Electron micrographic studies[5] have shown that the vanadocyte consists of distinct vacuoles (vanadophores) which house the vanadium, distinct from the nucleus[6,7] of the cell. It is known that the vanadium inside these vacuoles is maintained in the strongly reducing, air-sensitive, tripositive oxidation state at high concentrations (1.08 m in *Ascidia ceratodes*)[8] and at very low pH. The exact pH inside the vandophore is not known, but is certainly below 4 and may be much lower.[9] The remaining cytoplasm and nucleus of the vanadocyte, and the other blood cell types[6,7] in tunicate blood, are at near neutral pH and are bathed in oxygen-rich plasma.[10] Likely, the vanadium complex of tunicates is enclosed in the vanadophores to allow maintenance of low pH and strongly reducing conditions that would destroy the other physiological processes of the organism.

There are two approaches to studying the structure of this vanadium complex. The more classical approach has involved attempted isolation and purification of the vanadium complex as a prelude to determination of its structure. The difficulties in studying the vanadium complex isolated from lysed (not intact) cells are twofold. First, since the function of the vanadium complex is not known, there is no assay for establishing the integrity of the native complex throughout extraction and purification procedures preliminary to structural studies. Second, the complex present in vanadocyte hemolysates appears to be extremely unstable toward oxidation.[9] Therefore, it is perhaps not surprising that there are many conflicting conclusions concerning vanadium complexes in the hemolysate and that problems of artifact formation in hemolysates have been discussed often in the literature. The central conclusion of an earlier detailed study[11] of ascidian hemolysate involving magnetic susceptibility, UV–visible spectrophotometric, ultracentrifugation, oxidative potentiometric titration, and spectrofluorometric measurements addressed this problem directly and noted that "The properties of the vanadium in vanadocyte hemolysates do not give any conclusive information about the properties of vanadium in the much more condensed system inside the vanadocyte."

The alternative approach is to study the vanadium complex while it is still enclosed in the living cell. The UV–visible spectrum of living vanadocytes is unfortunately difficult to interpret, primarily because of the lack of specificity of the method. That is, any chromophore, organic or inorganic, inside the vanadocyte or not, can contribute to the UV–visible absorption spectrum. Indeed, recent reports[12-14] have suggested that all the features of vanadocyte UV-visible spectra are due exclusively to organic constituents, primarily tunichrome, a light-green compound with UV–visible absorption maxima at 325 and 660 nm (between pH 2.5 and 3.5). Recently, the fluorescence properties of ascidian whole blood cells have been studied and evidence has been found for a visible fluorescent material present within the vanadophores and likely associated with the membrane.[22]

The most revealing biophysical studies on intact cells have employed magnetic resonance methods. Results of EPR studies[15,16] have been primarily negative, because the V(III) ion normally shows no spectrum,[17] whereas VO^{2+} typically exhibits an intense eight-line spectrum.[18] Since intact frozen vanadocytes have been reported to exhibit no EPR signal,[15,16] the absence of VO^{2+} (at least for the two species studied) is indicated. In contrast, NMR experiments[8,16,19] have yielded several important findings concerning the structure of the native complex in living vanadocytes. Since V(III) has excellent NMR contact shift properties[20,21] (extremely short electron spin relaxation times and large hyperfine coupling constants), it acts like an in vivo "shift reagent", significantly shifting the NMR resonance of ligands to which it is bound without causing significant signal broadening. The NMR spectra of living *A. ceratodes*[8,19] and *A. nigra*[16] vanadocytes exhibit an intense Gaussian signal at 21 ppm. Detailed analyses[8,19] of the *A. ceratodes* living vandocyte NMR spectra demonstrated that the 21-ppm resonance is due to intravacuolar (vanadophore) water exchanging rapidly with coordination sites of the V(III) ions contained within the vanadophores. The 21-ppm signal disappeared upon cell lysis in a manner which suggested artifact formation.[8,19] The position of the 21-ppm resonance was shown to be a measure of V(III) coordination site concentration within the vanadophores. The spectra of numerous samples of living vanadocytes demonstrate that the resonance position of the 21-ppm peak varies only slightly, and therefore the concentration of V(III) within vanadophores is rigidly regulated by the cell (the mean concentration varying by only 0.02 m).[8,19] Since the number of vanadium ions in vanadophores could be measured by independent methods (atomic absorption), a measure of the total number of V(III) coordination sites [maximum of six per V(III) ion] was available. This number could be compared with the number of coordination sites available for exchange with the vanadophore water molecules. From these measurements it was determined that approximately two-thirds of the V(III) coordination sites must be available for coordination with vanadophore water molecules to result in a 21-ppm signal.[8,19] Conversely, two of the six coordination sites were suggested to be restricted from exchange with intravacuolar water molecules by some unknown ligand(s).

Since the NMR method does not give information on vanadium ligands other than water, or on metrical aspects of the structure, we have carried out further investigation of the coordination environment of the vanadium by X-ray absorption spectroscopy (XAS).[23] The element-specific nature of the XAS method allows direct study of the vanadium, and the high concentration of the metal ion in the vanadocytes makes measurements on intact, living cells possible. The ability of XAS to elucidate the coordination environment of metal ions in noncrystalline materials makes it, in general, a useful method for studying the structure of metal complexes in biological systems.[23-29]

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Accordingly, the XAS investigation reported herein encompasses three parts: first, a study of simple, structurally characterized compounds of vanadium to extend the application of XAS as a method of inorganic structure determination to vanadium complexes; second, determination of the structure of vanadium species in solutions that could serve as models for the milieu of the vanadophore [viz., V(III) in acidic solution]; and third, examination of vanadium in intact and lysed vanadocytes by XAS, using the results of the investigations above to establish the structures of these vanadium complexes.

Experimental Section

Materials. Vanadium Compounds. $VO(acac)_2$ was prepared by the method of Rowe and Jones.[30] $V(bpy)_3$ was prepared by the method of Herzog.[31] $VO(2\text{-Me-8-quinolinol})_2$ was prepared as described by Shiro and Fernando.[32] $V_2(SO_4)_3$ was prepared by the procedure of Claunch and Jones.[33] Tris(*cis*-1,2-diphenylethene-1,2-dithiolato)vanadium[34] [$V(S_2C_2Ph_2)_3$] was the gift of Professor R. H. Holm. $V[S_2P(OEt)_2]_3$ was prepared by the method of Furlani et al.[35] $(NH_4)_3VS_4$ was kindly provided by R. Chianelli of EXXON Research and Engineering Co. $V(acac)_3$ was purchased from ROC-RIC. VCl_3 was purchased from Alfa and $VOSO_4$ was purchased from K&K Laboratories. All other reagents were obtained from commercial suppliers and were used without further purification.

Air-sensitive vanadium(III) compounds and solutions were prepared and handled by using Schlenk techniques or in a dinitrogen inert atmosphere box (Vacuum Atmospheres). Visible spectra of V(III) solutions[36] were taken to monitor the presence of VO^{2+} contamination.

Vanadocyte Samples. Healthy specimens of *Ascidia ceratodes*[37] were collected from docks of the Monterey Marina in June and December 1977 and in February, April, May, and June 1978. The organisms were kept in fresh sea water until their blood was withdrawn, generally within 1 day of collection. The blood was removed[19] by shaving the tunic away from over the heart with a razor blade and then withdrawing the blood from the heart or branchial aorta at a slow rate with a disposable plastic syringe (Stylex tuberculin) fitted with a 25-gauge needle. Approximately 0.5 mL of blood was obtained from an average individual. The blood was placed in a centrifuge tube (12 mL) kept at 0 °C. The packed-cell XAS samples were prepared by centrifuging the combined blood of between 20 and 30 animals, using a desk-top centrifuge (2000 rpm, 3 min). The plasma supernatant was removed, and the lightly packed cells were taken up in a pipet and transferred to the XAS cell, a 7 × 2 × 0.08 cm piece of Lucite with a 3 × 0.6 × 0.08 cm window backed with Kapton (polyimide) film to hold the sample. The packed vanadocytes were layered uniformly on the Kapton and then covered by another piece of Kapton. The sample cell was maintained at +4 °C prior to recording the spectrum. The intact cell preparations were monitored for lysis, clearly indicated by the formation of dark patches characteristic of vanadocyte hemolysis in their own plasma. A sample was stable in the X-ray beam at room temperature for several hours, before it began to exhibit the characteristic color change associated with spontaneous vanadocyte lysis.

Lysed vanadocyte preparations were produced by subjecting a sample of packed *A. ceratodes* blood (centrifuged at 2000 rpm for 10 min) from which the plasma had been removed to several freeze–thaw cycles (liquid N_2/room temperature) until the sample was of a uniform deep red-brown color characteristic of spontaneously hemolyzed vanadocytes in a neutral medium. This preparation was then packed in an XAS cell as described above. A blue-green hemolysate was obtained by adding an excess of oxygenated sea water at near neutral pH to a sample of lysed cells prepared as above. Upon standing, a dark blue-green color formed in the cellular material, similar to the dark blue-green patches that form on the branchial sacs of unhealthy ascidia. The sea water was subsequently removed by centrifugation (2000 rpm, 10 min) and the dark blue-green pellet was loaded into the XAS sample cell.

Table I. Parameters for Vanadium EXAFS Curve Fitting (4–12 Å^{-1})[a]

	c_0	c_1	c_2	a_0	a_1	a_2	a_3
V–O	0.5962	−0.0207	1.2865	3.2079	−1.442	0.0385	
V–S	0.6155	−0.0190	1.1207	4.4844	−0.456		23.109
V–N	0.7232	−0.0134	1.8226	3.6132	−1.434	0.0308	

[a] Functional form (ref 23) used (for a single shell fit) is: $\chi(k) = C_0 e^{-c_1 k^2}/k^{c_2} \sin(a_0 + a_1 k + a_2 k^2 + a_3/k)$.

Table II. Vanadium Structure Determination. Comparison of EXAFS Curve Fitting with X-ray Crystallography

	EXAFS			diffraction		
	atom	R, Å	no.	no.	distance, Å	ΔR, Å
$VO(acac)_2$[a]	O	1.952	4.8	4	1.968	0.016
	O	1.587	1.0	1	1.571	0.016
VO^{2+} in acid[b]	O	1.568	0.7	1	1.559	0.009
	O	2.021	4.5	4	2.041	0.020
	O	2.235	1.3	1	2.284	0.049[c]
$VO(2\text{-Me-8-}$	O	1.592	1.3	1	1.600	0.008
$\text{quinolinol})_2$[d]	O	1.927	3.0	2	1.921	0.006
	N	2.139	2.6	2	2.136	0.003
$V[S_2P(OEt)_2]_3$[e]	S	2.476	4.8	6	2.451	0.025
$(NH_4)_3VS_4$[f]	S	2.15	3.1	4	2.15	0.000
					av ΔR	0.011

[a] See ref 46. [b] See ref 47a. [c] See text. [d] See ref 32. [e] See ref 35. [f] See ref 48.

Methods. XAS Data Collection. X-ray absorption spectra were measured at the Stanford Synchrotron Radiation Laboratory,[38] using monochromatic radiation from the SPEAR storage ring. During these experiments the energy of the storage ring beam was 1.8–1.9 GeV, with 20-mA average current, in the colliding beam mode. Extended X-ray absorption fine structure (EXAFS) spectra were obtained on EXAFS line 2, using a Si[111] two-crystal monochromator. High-resolution edge spectra were recorded on EXAFS line 1, using a Si[220] channel-cut crystal monochromator. Data were collected as previously described,[23,24a,26] in the transmission mode.

EXAFS Data Analysis Methods. The EXAFS was extracted from the X-ray absorption spectra and then analyzed by curve fitting, according to the methods we have previously described in detail.[23-26] The origin of the photoelectron wave vector, k, for vanadium spectra was taken to be 5490 eV. Vanadium spectra were calibrated by reference to a vanadium foil edge spectrum, with the first inflection point of the foil edge assigned as 5463.9 eV. A foil spectrum was taken several times every 8 h; the position of the inflection varied no more than a few monochromator steps, corresponding to less than 0.5 eV, over the course of 1 or 2 days.

$V(III)(acac)_3$,[39] $V(bpy)_3$,[40] and $V(S_2C_2Ph_2)_3$[41] were the single-shell model compounds whose spectra were analyzed to obtain V–O, V–N, and V–S parameters, respectively, for use in fitting the data of unknown compounds. The amplitude parameters for the oxygen scatterer (Table I) were derived from the Fe–O wave of $Fe(acac)_3$ data.[24a] This is reasonable since scatterer amplitude functions are transferable from one absorbing atom to another.[23,24a] This assumption was quantitatively tested. Curve fitting of data from numerous model V complexes, using amplitude parameters derived from $V(acac)_3$, gave unsatisfactory amplitude results, as did curve fitting for an acidic VCl_3 solution, using Table I parameters. Improved data for two V(III) solutions are being obtained. Good fits of the experimental data were obtained for all other compounds (results summarized in Table II) by using the phase and amplitude parameters in Table I.

In addition to the derived V–O, V–N, and V–S parameters, Table I includes the parameterized EXAFS equation[23] used to fit the experi-

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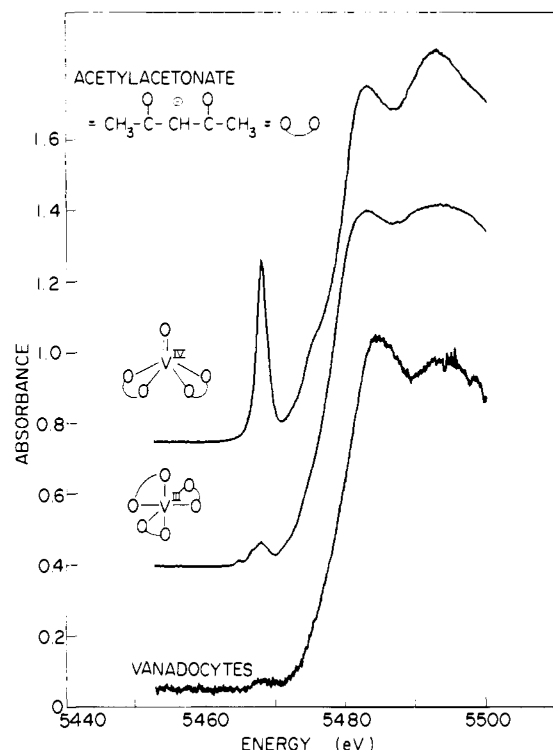

Figure 1. Vanadium edge spectra: $VO(acac)_2$, $V(acac)_3$, vanadocytes. The effect of the presence of an oxo group on the absorption edge is seen by comparing vanadium acetylacetonate. The large increase in intensity is due to extensive d-p mixing in the V=O molecular orbital, making the preedge transition at 5464 eV more allowed. The vanadocyte edge is very similar to the $V(acac)_3$ spectrum, indicating a symmetrical coordination environment for vanadium in the 3+ oxidation state in the living vanadocyte.

mental data. Two parameters (c_0 and a_1) per coordination shell were varied in the fits. The values of these adjustable parameters are related to the number of scattering atoms and their distances, respectively.

All data reduction and analysis was performed on a PDP 11/55 computer using the EXAFS Analysis Package (XAP), a set of interactive programs developed and evolved in our laboratories at Stanford.[26]

Results and Discussion

High-Resolution X-ray Absorption Edge Spectra. Edge spectra were recorded for 27 vanadium compounds of a variety of oxidation states (0, III, IV, and V), geometries, and ligand types. This catalog of edges[42] shows trends which are useful in characterizing the chemical state of vanadium.

The most striking feature of vanadium edges is the presence of an intense "preedge" transition if the vanadium has a terminal oxo group as a ligand (V=O). This phenomenon has also been noted in the edges of Mo=O complexes,[23,43,44] and is of diagnostic use for the presence of Mo=O species. The transition in V=O occurs at the energy of the typical high-symmetry vanadium bound state 1s to 3d preedge absorption (5468 eV), but its intensity is more than an order of magnitude greater. This higher intensity is due to mixing of the oxygen p levels with the metal 3d orbitals upon formation of the molecular orbital. Qualitatively, since s-d transitions are formally forbidden by dipole selection rules, while s-p are allowed, the added p character of the V=O molecular orbital makes this transition more allowed than it is for compounds of vanadium without this extensive d-p mixing.[45] As a result, it is a simple matter to determine if a vanadium compound contains a terminal oxo ligand. It should be noted that terminal sulfur (V=S) can give rise to an enhanced intensity preedge transition, as has been observed for molybdenum edges,[24a,43] but analysis of the EXAFS (as discussed below) can distinguish between V=O and V=S.

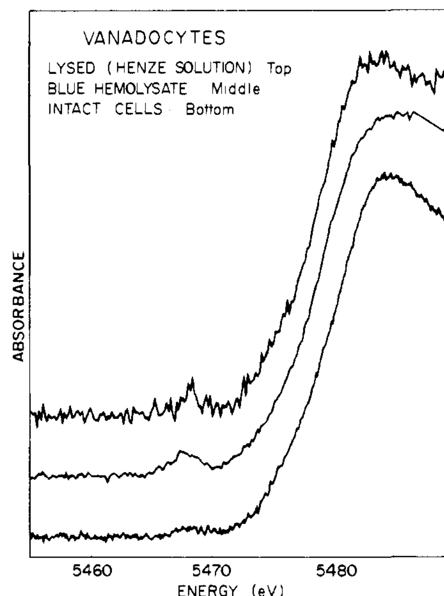

Figure 2. Comparison of X-ray absorption edges of intact and lysed vanadocytes. The lack of an intense preedge transition (as illustrated in Figure 1) in the lysed cell edges shows that there is remarkably little production of oxovanadium upon exposure of the V(III) of intact vanadocytes to air.

Figure 1 shows the K edge absorption spectra of $VO(acac)_2$, $V(acac)_3$, and intact vanadocytes. The vanadocyte edge most clearly resembles that of the symmetrically coordinated $V^{III}(acac)_3$, as both have the small preedge 1s to 3d ground-state transition. By contrast, the $VO(acac)_2$ edge exhibits a large preedge feature. This comparison is strong evidence for lack of any significant quantity of VO^{2+} in the living vanadocytes, confirming previous conclusions, based on magnetic susceptibility, redox titrimetric, spectrophotometric, EPR, and NMR measurements,[8,9,11,15,16,19] that the vanadium is in the III oxidation state in the living cells.

Perhaps less expected are the edge spectra of the spontaneously lysed vanadocyte preparations, compared in Figure 2 with the edge spectrum of the intact cells. If the vanadium were oxidized to VO^{2+} upon lysis of the vanadocyte, the preedge feature should be of much greater intensity for the lysed preparations. That there is at most a small increase in the size of this transition upon lysis demonstrates that only a small amount of VO^{2+} is present in the lysed cells or even in the blue hemolysate.

The intensity of the preedge transition is roughly proportional to the number of oxo groups present in the molecule (assuming similar energy resolution of the measurements being compared). For vanadium(IV) compounds with one oxo group, the transition is around 50% of the height of the main edge jump, while for vanadium(V) compounds with two short V-O bonds the preedge is about 80% of the main edge. By comparison, the normal preedge transition for vanadium(III) compounds having no oxo ligands is around 4% of the edge jump.

As a check on the sensitivity of the preedge transition to mixtures of V(III) and VO^{2+}, a solution of $V^{III}{}_2(SO_4)_3$ in aqueous $HClO_4$ was partially air-oxidized and its edge spectrum was recorded (Figure 3). The preedge is clearly intermediate in intensity when compared to edges of pure V(III) or VO^{2+} edges (Figure 1). The intensity of the preedge for the mixture corresponds to around 20% VO^{2+}; analysis of the UV-vis spectrum[36] indicated 24% VO^{2+}. Thus, from the edge spectra an upper limit can be placed of around 10% VO^{2+} in intact and in lysed vanadocytes (Figure 2).

The positions of inflection points and peaks in the edges of 27 vanadium compounds[42] were not found to be sufficiently sensitive

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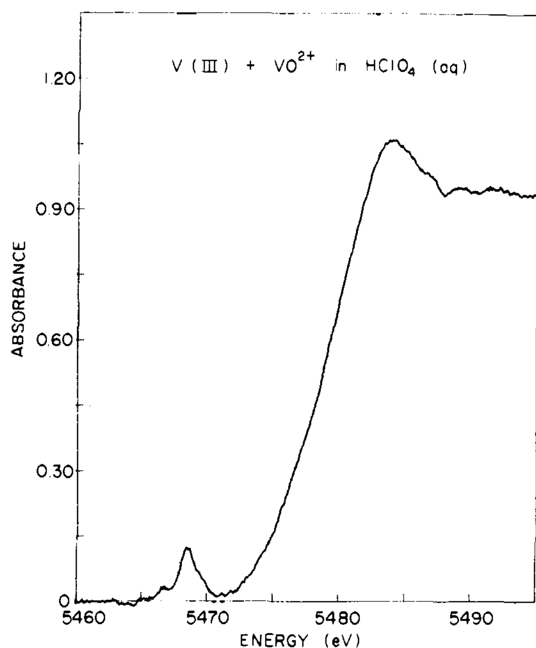

Figure 3. Edge of V(III) + VO^{2+}. This spectrum shows the sensitivity of X-ray absorption edges to mixtures of V(III) and oxovanadium(IV). The preedge transition is intermediate in intensity when compared to $V(acac)_3$ and $VO(acac)_2$ (Figure 1). The height of this peak corresponds to around 20% VO^{2+}; the UV-vis spectrum of this sample showed 24% VO^{2+}.

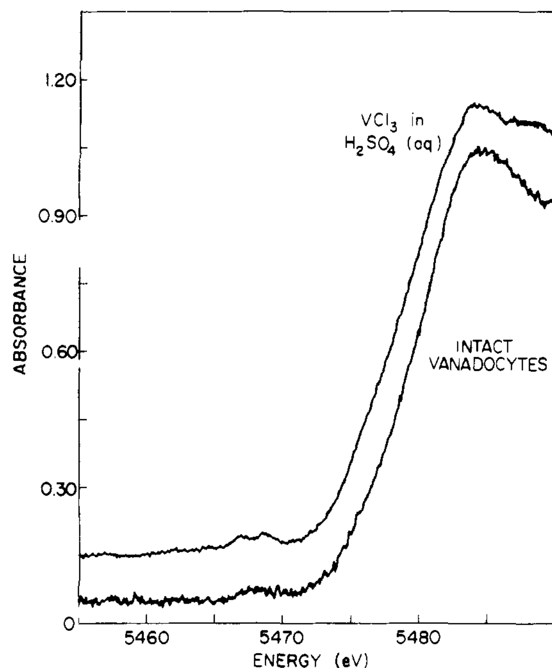

Figure 4. Edge spectra of vanadocytes and V^{3+} in acid. Even though vanadium edge positions do not correlate unambiguously with formal oxidation state, this comparison shows that the electronic structures of V^{3+} in acid and vanadium in vanadocytes are quite similar, even to the small splitting of the preedge 1s-3d transition. Rather high symmetry is possessed by both coordination spheres, based on the lack of intensity in the preedge transition.

to formal oxidation state to permit unambiguous assignment, by edge analysis only, of the formal oxidation state of vanadium in vanadocytes. However, the edge spectrum of vanadium(III) in aqueous sulfuric acid medium is identical with the spectrum of intact vanadocytes (Figure 4). Furthermore, the lack of structure superimposed on the main edge jump in the intact vanadocyte spectrum indicates a high symmetry environment for the vanadium. The presence of a small preedge transition rules out significant quantities of VO^{2+} (vide supra).

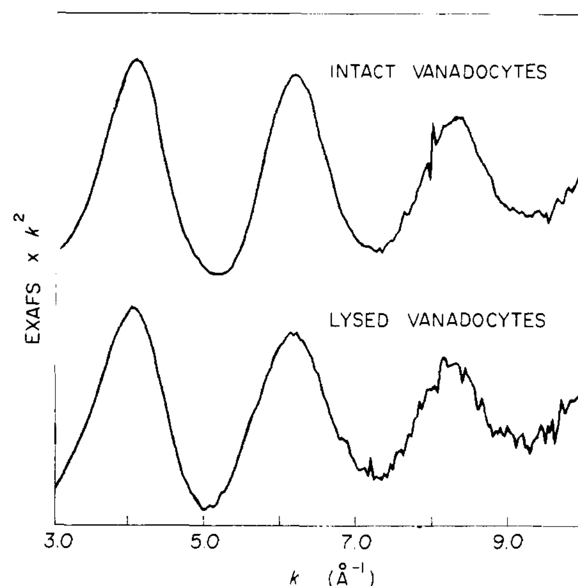

Figure 5. EXAFS of vanadocytes. A simple damped sine wave characterizes the EXAFS spectra of vanadocytes, both intact and lysed. The frequencies, phases, and amplitude envelopes are similar, indicating a common coordination environment. Of interest is the lack of change of the EXAFS upon lysis, corroborating the edge result (Figure 4) that little oxovanadium is produced upon spontaneous lysis. The EXAFS of the intact cells is of slightly higher amplitude, having been measured at 150 K. It has phase and amplitude envelope features identical, however, with those of the spectra of lysed cells run at ambient temperature.

EXAFS Analysis. The extended fine structure of the X-ray absorption spectrum can give much more detailed structural information[23] about the immediate coordination environment of vanadium. We have shown that, for molybdenum,[24a-c,28,43,44] copper,[26,27] and iron[25] complexes, curve-fitting analysis of EXAFS spectra offers metal–ligand bond distances with an accuracy of typically 0.02 Å and with less certainty can identify the type and number of ligands. To put vanadium EXAFS on a similar foundation, the EXAFS spectra of several structurally characterized vanadium compounds were obtained and analyzed by curve fitting.[23] These structure determinations give a measure of the confidence to be placed in the structure found by EXAFS for the vanadium complex of tunicates. Table II sets out the numerical results of our EXAFS structure determinations on the vanadium model compounds and compares the distances and coordination numbers obtained to those found by crystal structure analysis.[32,35,46-48] The agreement is good, with an average deviation in bond distance of 0.011 Å and coordination number determination to an accuracy of 25%.

The structure we have determined for VO^{2+} dissolved in acid deserves comment. Comparison of the crystal structures of either hydrated[47a] or anhydrous[47b] $VOSO_4$ with our solution EXAFS results (Table II) shows that the short oxo bond and the V–O bonds in the equatorial plane have the same lengths in both the solid state and in solution. The only difference in the coordination spheres is in the long V–O bond trans to the oxo group, which is 2.28 Å in the solid from crystallography and 2.23 Å in solution from EXAFS. Since such bonds (water ligand trans to oxo) can be highly variable in length, it is likely that crystal packing forces cause the difference observed. This structure determination of a complex ion in solution shows that the EXAFS is not adversely affected by chemical exchange. This is expected since the X-ray absorption time scale is much faster than exchange processes.

Having evaluated the accuracy of EXAFS curve fitting in the determination of a wide variety of vanadium coordination envi-

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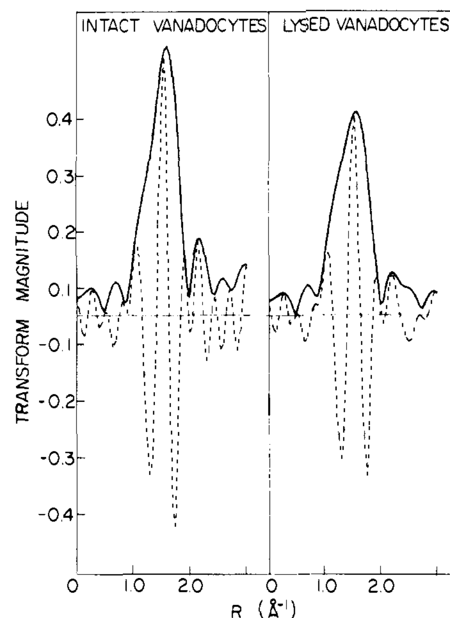

Figure 6. Fourier transforms of vanadocyte EXAFS. The transforms of the EXAFS data of Figure 5 again show the similarity among the vanadium environments in lysed and intact cells. The intact vanadocyte transform has a higher amplitude than the lysed cell transform because intact cell data were taken at a lower temperature. The EXAFS data were transformed[23] over 4–12 $Å^{-1}$.

Table III. Results of Single Oxygen Wave Fits of the EXAFS of Intact and Lysed Vanadocytes

vanadocytes	no. of oxygens	distance,[a] Å	F^b
intact (150 K)	5.4	1.99	0.424
lysed (room temp, red brown solution)	6.7[c]	1.99	0.418

[a] The errors estimated from the variance of a large number of comparisons of EXAFS results with known structures establish errors of about 0.02 Å in the bond distances and 25% in the coordination numbers. [b] $F = [\Sigma k^6(\text{data-fit})^2]^{1/2}$; see ref 23. [c] Relative Debye–Waller factor (c_1) allowed to vary, along with c_0 and a_1.

ronments, we could then investigate the structure of the vanadium in ascidian blood. The EXAFS spectrum of intact vanadocytes and the spectrum of lysed cells are shown in Figure 5. The main difference is that the intact cell spectrum is of slightly larger amplitude, because these data were taken at 150 K, while the other spectrum was measured at room temperature. Both spectra consist of a single damped sine wave, with no evidence for the superposition of more than one wave. The amplitude envelopes and frequencies are nearly indistinguishable, although the absolute amplitude difference exists due to temperature effects. Fourier transforms[49] of the data (Figure 6) confirm these observations. A single main peak is seen, with no ordered shells of scattering atoms at longer distance evident above the noise level. Curve-fitting analysis of Fourier-filtered data demonstrates that a single oxygen wave is sufficient to describe the EXAFS spectra (Figure 7). The distances and coordination numbers derived from the fits are compiled in Table III.

It is important to note that the curve-fitting method cannot distinguish between oxygen and nitrogen scattering atoms because the phase and amplitude characteristics of these two elements as scatterers are very similar (due to the similarity of their atomic numbers).[23,24,26] Thus, a nitrogen wave or a mixture of nitrogen and oxygen waves could as well provide a reasonably good fit to the vanadocyte EXAFS data. However, for vanadium in vanadocytes it is most likely that all the ligands are oxygen, rather than any being nitrogen, since the vanadium–ligand distance (1.99 Å) determined by EXAFS is similar to that found for other complexes of V(III) with oxygen ligands. Vanadium–nitrogen distances are usually significantly longer. Although there is some uncertainty in distinguishing oxygen and nitrogen scatterers, sulfur scatterers can be easily identified[23-25] because V–O and V–S waves are nearly π out of phase. The EXAFS thus rules out unequivocally the presence of any V–S bonds in the first coordination sphere of the vanadium in vanadocytes. In addition, there is no evidence for a short vanadium–vanadium interaction. The EXAFS results further reinforce the evidence from the edge studies that there are no terminal oxygen or sulfur ligands on the vanadium.

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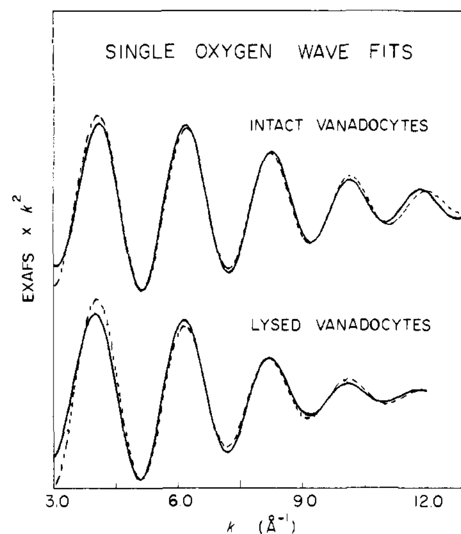


Figure 7. Curve fits of vanadocyte EXAFS. Single oxygen wave fits of the EXAFS data sets shown in Figure 5. The functional form used for these fits and the numerical results of the fits are given in Table III. The solid line is the Fourier filtered data and the dashed line is the fit.

Conclusions

The structure of the X-ray absorption edge of vanadium in living vanadocytes strongly supports the conclusion of earlier studies that the vanadium is in the tripositive oxidation state. Furthermore, the X-ray K absorption edge data indicate that the vanadium(III) is in a highly symmetrical coordination environment and put an upper limit (10%) on the quantity of VO^{2+} that can be present in living and in spontaneously lysed cell preparations. Analysis of the EXAFS from vanadocytes shows the presence of a symmetrical, single-shell coordination environment and provides strong evidence that all the first shell scatterers are of low atomic number, such as oxygen or nitrogen atoms. The EXAFS analysis clearly shows the absence of an ordered second shell of scatterers, indicating that most of the coordinated oxygen atoms are not bound to any atom other than hydrogen. These results agree with earlier NMR studies of living vanadocytes which demonstrated the presence of water molecules in the first coordination sphere of vanadocyte vanadium. The XAS results further suggest that all the coordination sites are occupied by oxygen. Since the vanadophore contains large quantities of sulfate ions, it seems reasonable to suggest that the nonwater oxygen-containing ligand is sulfate. The contribution to an EXAFS spectrum of a single second shell scatterer is much smaller,[25] so that, even if a single second shell sulfate sulfur atom were present in the vanadocyte vanadium complex, it would not necessarily be detected in the EXAFS spectrum. It is now clear from combined EXAFS and magnetic resonance data that the vanadium complex in living ascidian blood cells is a simple vanadium(III)–aquo complex which contains perhaps another small oxygen-containing ligand (possibly sulfate).

The riddle of the vanadocyte function remains as perplexing as ever. An important role of the structural results reported here will be to limit the possibilities for the function of the vanadium ion in tunicate blood cells. The vanadocyte shelters an exceedingly simple metal complex, the concentration of which it rigidly

maintains. This leads to the conjecture that the functional vanadium-containing species is in fact the vanadophore vacuole, or perhaps the entire vanadocyte, and not some smaller molecular unit.

Acknowledgments. We thank Patrick Frank for numerous helpful discussions and experimental assistance and Donald P. Abbott for help in the various biological aspects of this study. Partial support for R.M.K.C. was provided by NIH Grant GM 06840 to Carl Djerassi. The research was supported by the National Science Foundation through Grant PCM 79-0495 and the synchrotron beam time was provided by the Stanford Synchrotron Radiation Laboratory supported by National Science Foundation Grant DMR-77-27489 in cooperation with the Stanford Linear Accelerator Center and the U.S. Department of Energy.

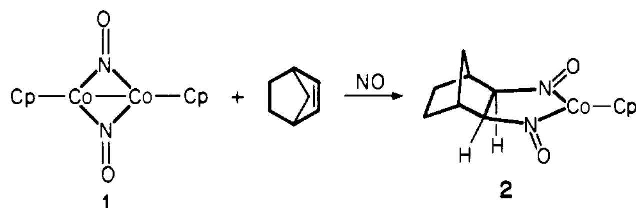
Communications to the Editor

A New Method for 1,2-Diamination of Alkenes Using Cyclopentadienylnitrosylcobalt Dimer/NO/$LiAlH_4$

Sir:

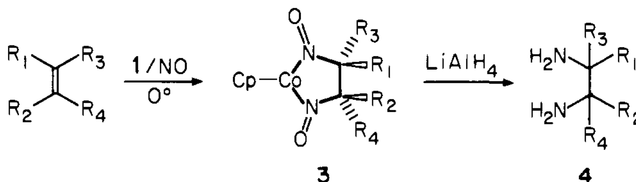
We report a new cobalt-based reagent for vicinal diamination of alkenes. This is the first general method for the direct conversion of olefins into primary vicinal diamines; because of this, and the fact that it works satisfactorily for terminal, *E* and *Z* di-, tri-, and at least some tetrasubstituted alkenes, it appears to circumvent the problems of scope encountered with reagents developed earlier.[1-3]

Our work utilizes some organometallic cobalt nitrosyl chemistry discovered by Brunner and Loskot.[4] These workers reported that the cobalt nitrosyl dimer **1**, easily prepared[5] by nitrosating



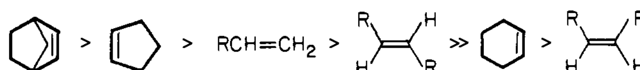
$CpCo(CO)_2$ ($Cp = \eta^5$-C_5H_5), reacted (in the presence of additional NO) with moderately strained bicyclic alkenes such as norbornene to give cis-exo complexes of general structure **2**. In the norbornene case, Bernal and his co-workers ultimately confirmed this structure by X-ray diffraction.[6] Brunner and Loskot noted that **2** reacted with both $LiAlH_4$ and I_2; however, the products of these reactions were not reported. They also observed the formation of cobalt alkylnitroso complexes similar to **2** when **1** was allowed to react with ethylene or cyclohexene and NO, but attempts to purify these materials were unsuccessful.

We have now observed that the reaction of **1** with ordinary alkenes is quite general, and the alkylnitroso complexes **3** so



generated can be stabilized (at least in solution) by performing the reactions at 0 °C rather than at room temperature. More significantly, in lieu of isolation, solutions of these adducts may be treated directly with $LiAlH_4$, leading on workup to primary vicinal diamines (**4**) in adequate to excellent *isolated* yield (Table I).[7]

Scheme I



The relative reactivity of alkenes toward dimer **1** increases with increasing ring strain. Interestingly, the relative reactivity of cis and trans isomers of a given alkene appears to parallel the palladium- and osmium-based reactions,[1,2] except that in our system the cis stereoisomers react rapidly enough to obtain product. A qualitative scale of reaction rates for a series of common alkenes is shown in Scheme I. This reactivity parallels the apparent thermal stability of the corresponding alkylnitroso complexes (**3**), the stereoselectivity of the $LiAlH_4$ reduction (see below), and the diamine yield.

The formation of pure cis-exo adduct **2** from norbornene suggested that the overall diamination might be stereospecific. Addition of the adduct solution *to* a suspension of $LiAlH_4$ in THF with rapid stirring gave reasonable stereoselectivity. In refluxing THF, the cis-exo diamine made up 84% of the mixture, and 10% and 6% of the other two isomers were found; reduction at −65 °C gave a 90:4:6 ratio.[8] Similarly, some stereoselectivity was observed with the other alkenes investigated by using this addition sequence (Table I).

The mechanism of this reaction is presently unclear, although the requirement for NO in the adduct-forming step suggests that $CpCo(NO)_2$ may be the initially formed intermediate. We have also found that treatment of **2** with 2 mol of I_2 in THF gives a complex whose structure is as yet unknown; however, when hydrolyzed with saturated aqueous $NaCl/Na_2S_2O_3$ solution, this material gives 2,3-dioxonorbornane dioxime in 60% yield. We are currently investigating the generality of this reaction.

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(7) The diamines are surprisingly easy to handle. They are quite volatile and may be purified by distillation or vapor-phase chromatography, and can be stored neat without very much decomposition over time. In most cases, diastereomeric diamines could be resolved by VPC (33 ft × 1/4 in. 10% DC 710 on Chrom W + 5% KOH 60–80 mesh); cf.: E. D. Smith and R. D. Radford, *Anal. Chem.*, **33**, 1160 (1961).
(8) This order of addition consistently gave the stereoselectivity indicated. Addition of $LiAlH_4$ *to* the solution of adduct **2**, however, has given more erratic stereochemical results. The ratios obtained in these experiments were sometimes reminiscent of those obtained on $LiAlH_4$ reduction of 2,3-dioxonorbornane dioxime. At the suggestion of a referee, we have carried out the reduction of **2** with $LiAlD_4$. Mass spectral analysis of the products showed substantial deuterium incorporation at the α-amino positions (for the *cis-exo*-diamine, we observed 52% d_0, 41% d_1, and 7% d_2 species; the two minor isomers, analyzed as a mixture, showed somewhat increased amounts of d_2 and decreased amounts of diamine-d_0. This indicates that at least part of the conversion to diamine (even in the cis-exo case) takes place after alkylnitroso → oxime isomerization of at least one of the C–N functionalities. It may be that some batches of $LiAlH_4$ contain Lewis acid species capable of promoting such rearrangement.

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