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VANADIUM: A BIOLOGICALLY RELEVANT ELEMENT

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I. Introduction

In recent studies vanadium has been shown to be an essential requirement in biological systems, and the chemistry of the element is

gaining considerable interest. This chapter focusses on the properties of the novel vanadium-containing bromoperoxidases. Since the chemistry of vanadium is of direct relevance to the mechanism of action of bromoperoxidases, the chemistry of vanadate, properties of vanadium(V) complexes and their reactivity, including reactions with peroxide, will be discussed. Further, the work in three selected areas, vanadium in mushrooms, vanadium in oil and coal, and vanadium in tunicates, will be reviewed. No attention will be paid to the effect of vanadate on enzyme systems that are inhibited or activated due to the parallelism in chemistry with phosphate. For this and for the general chemistry of the element the reader is referred to a number of reviews. (*1–5*). Work on the vanadium containing nitrogenases will not be covered here. For studies concerning this enzyme and information to its active site structure, the reader should see Ref. *6* and references therein.

II. Vanadium Bromoperoxidases

A. Discovery

The presence of iodoperoxidase and bromoperoxidase activity in seaweeds has been well documented since the beginning of the 20th century (*6–8*). However, little interest was paid to these enzymes till the 1980s. Most people assumed that these peroxidases were hemoproteins, similar to those seen in granulocytes, the thyroid gland, milk, saliva, yeast, fungi, and horseradish (for a review see *9*). The reason for this was that the marine red algae *Bonnemaisonia hamifera* (*10*) and *Cystoclonium purpureum* (*11*) and the marine green alga *Penicillus capitatus* (*12*) contained a bromoperoxidase with a heme prosthetic group.

A novel class of haloperoxidases, in which a heme prosthetic group was absent, was detected in brown algae (Phaeophyceae) by Vilter and coworkers (*13–15*). These publications escaped the attention of most biochemists involved in peroxidase research. Only when some of this work was published in the journal *Phytochemistry* (*16*) was there as increasing awareness of these findings. A clue for the involvement of vanadium was also published (*16*). It was shown that the bromoperoxidase could be inactivated at low pH and reactivated by vanadate. These results were subsequently confirmed (*17, 18*) when it was shown that vanadium was present in a number of bromoperoxidases from different sources and was essential for enzymatic activity. To date, these sources include the enzymes from the brown seaweed *Ascophyllum nodosum*

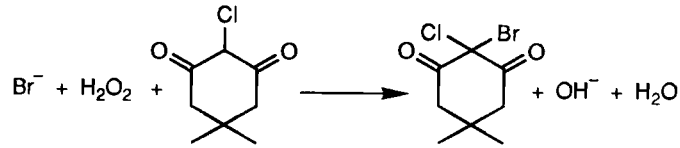
(knotted wrack) (*19, 20*), *Laminaria saccharina* (sugar kelp) (*21*), and *Chorda filum* (*21*).

Furthermore, vanadium-dependent iodoperoxidase was detected (*21*) in *Fucus spiralis, F. serratus, F. vesiculosis,* and *Pelvetia canaliculata,* in line with ref. *12*. Since most peroxidases are able to oxidize iodide, and little is known about the vanadium-containing iodoperoxidases, they will not be discussed here. Bromoperoxidases were subsequently discovered in the red seaweed (Rhodophytaea) *Ceramium rubrum* (*22*) and in a terrestrial organism, the lichen *Xanthoria parietina* (*23*). This lichen grows on rocks and stones and is a symbiosis of a green alga and a fungus. The role of the enzymes in the seaweeds has not been firmly established, but it is generally assumed that these enzymes are involved in the biosynthesis of bromometabolites. Marine algae are a rich source of a fascinating diversity of these metabolites (*24*). For example the seaweed *A. nodosum* has been reported to produce huge amounts of volatile halogenated compounds such as CH_2Br_2, $CHBr_2Cl$ and $CHBr_3$ (*25*).

Vanadium is also an essential element for some marine macro-algae, such as the brown seaweed *F. spiralus* and the green seaweed *Enteromorpha compressa*. The growth yield of these marine algae is enhanced considerably (*26*) when vanadate is added to the culture medium, which consists of artificial seawater. Some seaweeds contain vanadium. A study of 70 seaweeds from Japanese coastal waters yielded vanadium contents ranging from 0.3 to 10.6 ppm on the basis of dry weight (*27*).

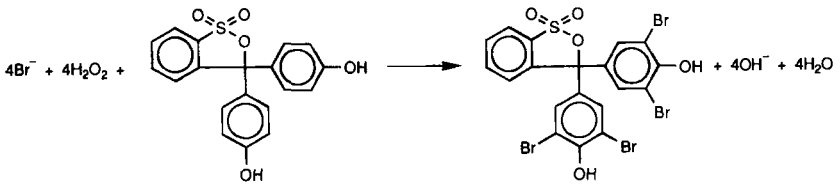
B. Detection of Bromoperoxidase Activity

Most assay methods to detect bromoperoxidase activity are based on the bromination of monochlorodimedone, a cyclic diketone that has a high affinity for HOBr and that on bromination loses it absorbance at 290 nm ($\varepsilon = 20.2\ mM^{-1}\ cm^{-1}$). This assay method was originally developed for measurements of chloroperoxidase activity by Hager et al. (*28*).



(1)

Alternatively, bromination of phenol red to bromophenol blue may be used (*29*).



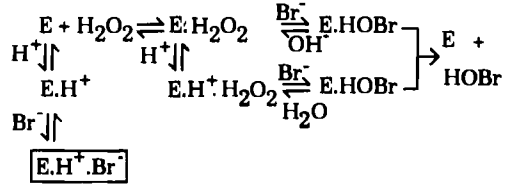
(2)

A further reaction involves the bromination of fluoresceine to the tetrabrominated compound eosine (*30*). These are convenient assays, since marked color changes take place.

Surprisingly, the bromoperoxidases do not (like other peroxidases) oxidize organic electron donors (*19, 31*) such as o-dianisidine or guaiacol, which are normally used to detect peroxidase activity, and thus enzymic activity easily escapes detection. When bromide or iodide are present in the assay medium HOBr and HOI are formed; these reactive species will oxidize, brominate, or iodinate the organic dyes, thus allowing detection. Early in this century similar organic dyes were used to detect peroxidase activity in seaweed tissues and extracts. However, the results were not clearly reproducible. This is understandable since in seaweed variable amounts of iodide and bromide are present. On purification of the bromoperoxidase the halides are removed, resulting in an apparent decrease in or lack of activity.

C. Kinetic Properties and Reaction Mechanism

The steady-state kinetics of the reaction of vanadium bromoperoxidase with hydrogen peroxide and bromide have been extensively studied (*19, 32, 33*). The results show that hydrogen peroxide reacts first with the enzyme and that bromide is the second substrate in the catalytic cycle. Measurement (*33*) of the rate of enzymic production of oxidized bromine species (Br_2, Br_3^-, and HOBr), as well as of the rate of bromination of 2-chlorodimedone, 5-phenylbarbituric acid, 2-thiouracil, and trans-4-hydroxycinnamic acid have shown that a ternary complex between the enzyme-halogenating intermediate and the organic substrate is not involved. This was also concluded from a study of the bromination of several barbituric acid derivatives and, together with the lack of stereoselective bromination (*34*), suggests a mechanism in which the oxidized bromine species are released into solution by the enzyme in a rate-determining step. This requires, however, that the enzyme is not inactivated by any of the products. Indeed, the vanadium bromoperoxidase was shown (*31*) to be resistant against 0.5 mM HOBr.



SCHEME 1. Simplified mechanism of action of vanadium bromoperoxidase. The species in boxes represent inhibited forms of the enzyme.

In contrast, the fungal heme-containing chloroperoxidase from *Caldariomyces fumago* is rapidly inactivated by HOBr (*31*). As early as 1926 Sauvageau (*30*) concluded that certain species of seaweeds are able to produce free bromine in solution. Scheme I gives the tentative reaction mechanism of bromoperoxidase with its substrates (*32, 34*).

The exact nature of the brominating species released by the enzyme is unknown. Hypobromous acid is known (*35*) to be in rapid equilibrium with molecular bromine and tribromide ions in aqueous solutions. Further, it is important to note that at neutral pH values a very fast reaction occurs between H_2O_2 and OBr^- to yield singlet oxygen (*36*). This rapid side reaction hampers direct observation of brominating species at neutral pH values and is why rapid-reaction kinetics had to be used by De Boer and Wever (*33*) to detect Br_3^- formation.

The occurrence of this side reaction is one of the reasons why at low concentrations of nucleophilic acceptors the bromination rate of the organic compounds showed (*33, 34*) a hyperbolic relationship with respect to concentration. At first sight this points to a specific interaction between an enzyme-halogenating intermediate and the nucleophilic acceptor. However, at high concentrations of the nucleophilic acceptors the same rate of bromination was observed and, further, the apparent K_m for the nucleophile increased at higher concentrations of H_2O_2 (*34*). These observations suggest the existence of two reactions in which the nucleophilic acceptor (AH) and H_2O_2 compete for HOBr:

$$H_2O_2 + HOBr \rightarrow O_2 + Br^- + H_2O + H^+ \quad (3)$$

$$AH + HOBr \rightarrow ABr + H_2O \quad (4)$$

Interestingly, unlike the heme-containing peroxidases myeloperoxidase and chloroperoxidase, the vanadium enzyme does not catalyze the direct disproportionation of H_2O_2 in the absence of bromide or iodide

(*33, 37*). In the presence of these halides, catalase activity is observed which, according to Everett and Butler (*37*), may be due to reaction of H_2O_2 with an enzyme-bound OBr^- moiety, an enzyme-bound Br moiety, HOBr, or Br_3^-. However, for catalase activity the reduction of H_2O_2 by H_2O_2 is required and this step is not observed. The available data suggest that H_2O_2 reacts with the enzymically generated oxidized bromine species in solution, according to Eq. (3).

All these vanadium-containing enzymes exhibit a pH profile with optimum pH ranging from 5.5 for the enzyme from the lichen *X. parietina* to 7.4 for the enzyme from the red seaweed *C. rubrum* (*22*). The pH study of De Boer and Wever (*33*) showed that inhibition of enzymic activity at low pH is due to protonation of an ionizable group, and that H_2O_2 is unable to bind to the native enzyme when this group is in a protonated state. The pK_a for this functional group controlling the binding of H_2O_2 (second-order rate constant 2.5×10^6 M^{-1} s^{-1} at pH>6) was 5.7, and was ascribed to a histidine residue. However, protonation of a water molecule bound to the active site cannot be excluded. EPR studies (*38*) on reduced bromoperoxidase also showed the presence of an acid/base group with a pK_a of 5.4. It is conceivable that this group is responsible for the pH-controlled binding of H_2O_2.

The bromide ion does not appear to react with one form of the enzyme-hydrogen peroxide complex. It is clear that at least two pH-dependent intermediates are present, which react with bromide to yield the oxidized bromine species. The second-order rate constant for the reaction between bromide and these peroxo-intermediates was estimated to be 1.7×10^5 M^{-1} s^{-1}. Bromide also acts as an inhibitor of the enzyme in a complex fashion (*19, 33*). Thus maximum inhibition is observed (K_i = 228 mM) at pH 5.2, whereas at higher and lower pH values little of the inhibitory complex is formed. The complex inhibition pattern and pH dependence of the binding of substrate is probably responsible for the skew (*19, 33*) as opposed to the bell-shaped curve for activity versus pH.

Unlike the findings with most heme-containing peroxidases, no inhibition is observed (*19, 33*) at high concentrations of H_2O_2. Therefore high concentrations of H_2O_2 do not damage the enzyme, and nonproductive intermediates are not formed. Also, Cl^- had no effect on the enzymic activity (*19, 37*) of bromoperoxidase. From a physiological point of view and considering the concentration of chloride in seawater (0.5 M) the latter observation is understandable. Inhibitors such as azide and cyanide, which normally inhibit the activity of the hemeproteins, have no significant effect on the enzymic activity of the vanadium bromoperoxidases (*20–22*). It should be noted, however, that

the apparent inhibition of the bromination of 2-chlorodimedone by cyanide is due to a competitive reaction of HOBr with CN^- to yield CNBr (*39*).

For the vanadium bromoperoxidase from the lichen *X. parietina* it has been reported (*23*) that an excess (50 mM) of F^-, Cl^-, and Br^- inhibited the enzymic activity. This enzyme from the terrestrial organism also had a surprisingly high affinity for bromide (K_m = 0.03 mM), which is about two orders of magnitude higher than the values reported for the enzymes from seaweed (*19, 22, 32, 33*). This particular enzyme was also inhibited by low concentrations (1–5 mM) of nitrate (*32*).

An important question is whether during catalysis the oxidation state shuttles between vanadium(IV) and (V). From EPR and EXAFS data, which are considered later, it has been concluded that isolated vanadium bromoperoxidase contains vanadium in oxidation state V (d^0). De Boer et al. (*21*) have suggested that vanadium(V) serves as the binding site for substrate hydrogen peroxide and bromide. This notion is supported by the observation that hydrogen peroxide (alone or in combination with bromide) is unable to rapidly oxidize vanadium(IV) bromoperoxidase (*32, 38*). Furthermore, hydrogen peroxide and bromide were unable to reduce native bromoperoxidase to the vanadium(IV) state (*20*). These data support a model in which no redox changes occur during catalysis. That the metal ion serves as a binding site for peroxide is amply supported by the well-known property of inorganic vanadium(V) compounds to form stable peroxovanadium(V) complexes with hydrogen peroxide.

D. General Properties

According to SDS-polyacrylamide gel electrophoresis, the vanadium bromoperoxidases contain subunits with a molecular mass of about 65 *k*Da (*21, 23, 32*). However, there is a yet unexplained discrepancy with the molecular mass of native bromoperoxidase from *A. nodosum*. From HPLC measurements (*20*) and reconstitution experiments (*38*), a molecular mass of about 90 kDa is indicated.

Some vanadium-containing bromoperoxidases, such as that from *A. nodosum*, exhibit a remarkable chemical stability (*19, 29, 32*). For example, the enzyme from *A. nodosum* remains fully active in media containing appreciable amounts of methanol, ethanol, propanol, or butanol (up to 60% v/v). In these experiments the effect of solvent on the initial rate of bromination was studied. When the enzyme was stored in acetone, methanol, and ethanol, stability was observed for more than one month. Also, when the enzyme was kept under turnover conditions

in an enzyme reactor in the presence of substrate and phenol red (to scavenge HOBr), the enzyme remained active for three weeks (*29*).

Some of the vanadium enzymes are thermostable (*19*), although not as much as enzymes in thermophilic bacteria (*40*). For example, the enzyme from the red seaweed *C. rubrum* (*22*) is less stable than that from the brown seaweed *A. nodosum*. This may be related to the habitat of the seaweeds, since red seaweeds remain submerged even at low tide. In contrast, *A. nodosum*, which is found near the high-tide level, is dry most of the time and in summer is exposed to considerable heat. Similarly, the enzyme from *X. parietina*, a lichen that grows on sun-exposed stones, has been reported to be thermostable (*23*).

The enzyme from *A. nodosum* is also resistant towards denaturation. Incubation of the enzyme in 1% SDS does not affect the enzymic activity; it is possible to detect brominating activity on the gel after SDS-gel electrophoresis (*19*). Only when the enzyme is boiled for a few minutes in SDS and β-mercaptoethanol is activity lost. Thus, this class of enzymes has remarkable properties and appears to be the first example of an oxidoreductase having such high stability.

E. EPR Properties

When native bromoperoxidase is reduced with sodium dithionite and then frozen, an EPR signal is observed (Fig. 1) that is assigned to

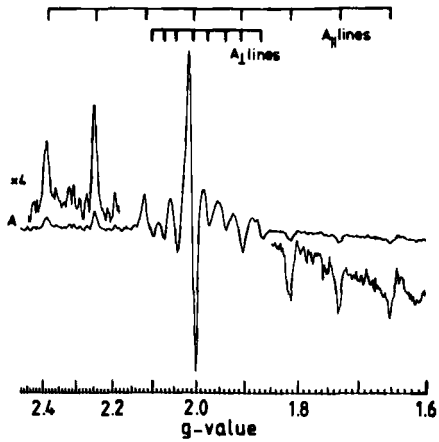


FIG. 1. EPR spectrum of reduced bromoperoxidase from *L. saccharina*. Instrument setting: microwave frequency 9.2 GHz, microwave power 20 dB, modulation width 1.0 mT; temperature 50K.

oxo-vanadium(IV) ligated to the protein. Two sets of eight hyper-fine lines in an axially symmetric EPR signal are present, due to coupling of the unpaired electron with the nuclear moment of ^{51}V (I = 7/2).

As can be seen from Table I, the enzymes from various species show the same EPR parameters, indicating that the structure and ligands coordinating to the vanadium(IV) species are similar. These EPR spectra are pH-dependent, the parameters $g_{\parallel}$ and $g_{\perp}$ hardly change whereas $A_{\parallel}$ and $A_{\perp}$ show a considerable increase on lowering the pH. Since $A_{\parallel}$ and $A_{\perp}$ are sensitive measures of the coordination environment (*42–45*), it is concluded that the coordination of vanadium(IV) is affected by pH.

Correlations of EPR data such as g_0 versus A_0, and $g_{\parallel}$ versus $A_{\parallel}$, have been used to estimate the average ligand environment of the vanadium(IV) species (*42–45*). Comparison of the data in Table I with such plots suggests that the ligand environment consists largely of oxygen and/or nitrogen donor atoms.

From studies on the pH dependence of EPR signals, a pK_a of 5.4 has been calculated, which suggests (*38*) protonation of either histidine or aspartate/glutamate residue near the metal site. There is a significant decrease in hyperfine line width when the enzyme is dissolved in D_2O. Also when $H_2{}^{17}O$ is used, the line width is affected (*38*). This demonstrates that water forms part of the coordination environment of the vanadium(IV) ion. In D_2O the resolution of EPR spectra is greatly

TABLE I

EPR PARAMETERS OF REDUCED BROMOPEROXIDASES

Species	Experimental conditions	$g_{\parallel}$	$g_{\perp}$	g_0	$A_{\parallel}^a$	$A_{\perp}$	A_0	Reference
saccharina	0.1 M Tris-SO_4 (pH 8.3)	1.948	1.979	1.969	164.5	53.6	90.7	*21*
parietina	0.1 M Tris-SO_4 (pH 8.3)	1.945	1.979	—	165.4	54.2	—	*41*
nodosum	0.1 M Na-citrate (pH 8.3)	1.948	1.979	1.969	160.1	50.2	86.6	*38*
nodosum	0.1 M Na-acetate (pH 4.2)	1.953	1.981	1.967	165.7	54.2	91.6	*38*

[a] A values listed in units of $10^{-4}cm^{-1}$.

increased, and a small in-plane anisotropy becomes visible (*38*), which may suggest some mixed-ligand coordination. The fact that protons have access to the oxo-vanadium(IV) ion in reduced bromoperoxidase was also demonstrated by electron spin echo envelope modulation spectroscopy (ESEEM) of the reduced bromoperoxidase (*46*). The Fourier transform of ESEEM spectra shows an ^{1}H modulation at 13.8 MHz, which is replaced by a deuterium modulation at about 2 MHz when the experiment is carried out in D_2O. Furthermore, it was concluded (*46*) from a comparison of ESEEM spectra of bromoperoxidase with those of a number of model complexes, that nitrogen (^{14}N frequencies at 3.1, 4.2, 5.3, and 8.1 MHz) is present in the equatorial plane of the oxo-vanadium(IV) of reduced bromoperoxidase. However, the possibility that the observed nuclear modulation is due to coupling of a nearby nitrogen atom with the paramagnetic center via a coordinating oxygen atom cannot be excluded.

F. XANES AND EXAFS

Vanadium K-edge X-ray absorption spectra have been reported for the enzyme in its oxidized and reduced forms (*47–49*). From a comparison of the energy position of the pre-edge feature in a number of model complexes, which is directly related to the coordination charge of the metal (*50*), it is concluded that the vanadium in the native enzyme has an oxidation state of V. On reduction, vanadium(IV) is formed, as already indicated from EPR experiments (*20, 32, 38*). The two research groups involved have also reported experiments in which the substrates (H_2O_2 and bromide) were added to the enzyme (*47–49*). For the enzyme treated with H_2O_2 a small decrease in metal charge was observed, which would suggest coordination of H_2O_2 to vanadium. However, these results were not confirmed by Arber et al. (*48*). In this respect it should be noted that Hormes et al. (*47*) used freeze-dried samples, whereas Arber et al. (*48, 49*) studied the enzyme in a frozen solution. Hormes et al. (*47*) arrived at the conclusion that the vanadium in native bromoperoxidase is solely coordinated by oxygen donor atoms and that the coordination sphere has the symmetry of a distorted octahedron. Furthermore, on reduction of the enzyme a substantial change in coordination number was indicated. More detailed structures have been proposed by Arber et al. (*48*) on the basis of EXAFS spectra of both reduced and native enzyme. The EXAFS spectra of the two species differ considerably, which supports the idea that on reduction a significant change in the vanadium environment takes place. In the reduced enzyme the dominant contribution is from light atom backscattering at

about 2 Å, whereas other contributions occur at 3 Å and 4.3 Å. The effects at longer distances may result from scattering from groups such as imidazole or tyrosine. As already discussed, ESEEM (*46*) data point to an equatorial nitrogen atom derived from a histidine.

For reduced bromoperoxidase the following distorted octahedral geometry was proposed: two imidazoles at 2.11 Å, one oxygen at 1.63 Å, and three oxygens at 1.91 Å. The short oxygen bond probably corresponds to that which dominates the ligand field in the EPR spectra of the reduced enzyme (*17, 19, 32, 38*). De Boer et al. (*38*) showed that, when reduced bromoperoxidase was dissolved in water containing oxygen-17, the vanadium(IV) hyperfine line width was markedly increased. This increase arises from the coupling of the unpaired electron with the nuclear spin of ^{17}O and suggests that water is also present in the coordination sphere of the vanadium atom. The EXAFS data of native bromoperoxidase are consistent with one oxygen at 1.61 Å, three oxygen atoms at 1.72 Å, and two nitrogen atoms at 2.11 Å. Since EXAFS cannot discriminate between nitrogen and oxygen, it may also be that one or two oxygen atoms instead of nitrogen are present at 2.11 Å. It is conceivable that one of these oxygen atoms is derived from water. The short vanadium–oxygen distances are typically similar to those seen in vanadate or vanadium(V)-alkoxy systems (*51, 52*). Figure 2 shows the structure of the active site as present in bromoperoxidase (*48*).

G. Inactivation and Reconstruction

One of the properties of the prosthetic group in vanadium bromoperoxidase is that it can be removed at low pH (rendering the enzyme inactive), by dialysis against 0.1-M citrate-phosphate buffer con-

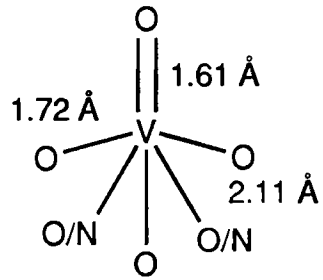


Fig. 2. Proposed structure for the active site in vanadium bromoperoxidase according to Ref. *48* and slightly modified.

taining EDTA at low pH. As originally discovered by Vilter (*16*) and since confirmed (*19*), the brominating activity of the apo-enzyme in Tris buffer (pH 8.3) can be restored by addition of vanadate (VO_4^{3-}). It has been shown that the presence of phosphate (PO_4^{3-}) accelerates the process of inactivation (*41*). Similarly, it has been observed that inactivation of bromoperoxidase occurs more rapidly in phosphate buffers than in Tris buffers. This may be related to the observation that phosphate catalyzes the hydrolysis of vanadate esters and diesters (*53*). It has been shown by de Boer et al. (*20, 38*) that phosphate and molybdate (MoO_4^{2-}) also inhibit the reconstitution by vanadate, and it was concluded that molybdate and vanadate compete for the same site on the enzyme. From studies on the reactivation process between apo-enzyme and vanadate, Vilter (*16*) has obtained a dissociation constant of 35 nM. De Boer et al. (*30*) also observed that vanadate has a high affinity for the apo-enzyme.

These observations have important implications for the structure of the active site. It is likely that on reincorporation of vanadate (VO_4^{3-}) into the apo-enzyme the four oxygen atoms are retained. This suggests that at least four oxygen atoms are present in the coordination sphere of the vanadium atom. Recent ^{51}V NMR experiments by Vilter and Rehder on vanadium bromoperoxidase showed an unusual ^{51}V chemical shift (*54, 55*), and they suggested coordination of six to seven highly electronegative oxygen functions to the vanadium atom. This proposal is in line with the XANES (*47*) study of native enzyme. However, EXAFS (*48*) and ESEEM (*46*) experiments on the reduced enzyme point to four to five oxygen atoms and at least one nitrogen atom.

H. Vanadate Complex Formation

An important question is, of course, how the vanadate is bound or coordinated to amino acid residues or other groups in bromoperoxidase. It is likely that a reversible ester bond (or bonds) with OH groups derived from the enzyme is (are) formed.

The formation of vanadate esters with hydroxyl groups in aqueous solutions has been studied in detail for methanol (*53*), ethylene glycol (*57*), phenol, and tyrosine (*58*). Vanadate is able to form cyclic complexes when there are adjacent hydroxyls in the molecule (*59*) and interacts with uridine, adenosine monophosphate (*60*), glutathione disulphide (*61*), and phosphate (*62*). Rehder (*63*) studied the interaction of amino acids and dipeptides with vanadate. He concluded that complexes are formed in which the peptide function and the *N*-terminal amino group are involved. Similarly, it was reported (*64*) that vanadate

binds to the protonated amino groups of glutathione and histidine. Side chains of amino acids may also participate; for example, the alcoholic function of serine may take part in coordination to vanadium. Another function that is principally available for interaction with the vanadate ion is a carboxylate. No evidence has been found for vanadate species containing the carboxylate ligand in the case of the amino acids glycine and aspartic acid (*63*). These findings are in contrast to those by Tracey et al. (*65*), who showed that vanadate forms derivatives with oxalate, lactate, and glycerate in which the carboxylate is clearly involved.

EXAFS data on bromoperoxidase (*48*) point to backscattering from light atoms, which may be due to histidine coordination or the presence of a tyrosine. However, intense ligand-to-metal charge-transfer bands in the visible spectra, such as those seen in mono-oxovanadium(V) phenolates (*66–69*), have not been observed in bromoperoxidase (*15, 16, 19*). Thus, the proposal (*48*) of a tyrosine residue coordinated to vanadium(V) in bromoperoxidase is not very likely. In fact, the tailing observed (*15, 16, 19*) in the absorbance spectrum of bromoperoxidase from 280 to 350 nm may be due to the absorbance of vanadate (*56*) incorporated in the enzyme.

A rather detailed study of the interaction of vanadate with amino derivatives was published recently by Crans and Shin (*70*). They showed that ethanolamine derivatives will form complexes with vanadate when their third and/or fourth functionality is an alcohol, a carboxylic acid, a phosphonium acid, or an amine. The central functionality should be a nitrogen atom with an available lone pair and the amine should be substituted by at least two ethyl arms. Increasing the substitution on the nitrogen stabilizes the complex; this suggests that a hydrophobic environment might favor the stability of these complexes. Although some of the reported complexes had a dissociation constant in the milli-molar range, this is still three orders of magnitude higher than those reported for the equilibrium between apobromoperoxidase and vanadate. They also showed (*70*) that Tris will also form weak complexes with vanadate and therefore preferably should not be used in biological studies.

I. Stoichiometry

It is consistently observed that the amount of vanadium in various bromoperoxidase preparations and from various sources is less than stoichiometric (*20, 21, 23, 38*). This may be due to loss of the metal during purification under rigorous conditions. However, it may also be that in the seaweed the bromoperoxidase is already present as apobro-

moperoxidase. The concentration in seawater of vanadium as vanadate is about 50 nM (*71, 72*). The value of the dissociation constant for the equilibrium between apo-enzyme and vanadate is 35 nM (*16*) and this suggests that if no specific mechanism is present by which the seaweed accumulates vanadium, part of the enzyme is in the apo-form.

III. Catalytic Activity of Some Vanadium Peroxo Complexes

A question that must be answered is why nature has chosen vanadium as a prosthetic group in bromoperoxidases. As pointed out by Djordjevic (*73*), the electron-rich peroxide group will easily form complexes with metal ions of low d^n configuration, such as molybdenum and vanadium. As will be discussed briefly, a number of these complexes are good oxidants, and the oxidizing properties of vanadium(V) peroxo complexes are directly relevant to the way in which the vanadium-peroxo complex in bromoperoxidase is able to oxidize bromide to hypobromous acid. Mimoun et al. (*74*) have shown that vanadium-peroxo complexes are effective oxidants in nonprotic solvents under mild conditions; these complexes not only catalyze the epoxidation of olefins to epoxides, but also hydroxylate aromatic hydrocarbons and alkanes to alcohols and ketones in nonselective manner. From a study on the hydroxylation of aromatic hydrocarbons to phenolic compounds it was concluded (*74*) that these complexes are able to transfer oxygen to aromatic hydrocarbons. However, alkanes are much less readily hydroxylated by V(V) peroxo complexes than aromatic hydrocarbons, and mixtures of alcohols and ketones are formed. The mechanism of oxygen transfer from peroxo-metal complexes to nucleophilic substrates is a matter of considerable debate [see Campestrini et al. (*75*) and references therein]. Two alternatives have been proposed. One proceeds through a simple bimolecular mechanism involving the nucleophile and the peroxo-metal complex. The other is a mechanism (*76*) that involves formation of an intermediate resulting from coordination of the substrate to the metal. A model indicating how metal-bound peroxo anions can transfer oxygen to the nucleophilic alkanes is shown (*77*).

Selective epoxidation of olefins by vanadium(V) alkyl peroxo complexes has also been reported (*76*). These complexes are very effective stereo-selective reagents for the transformation of olefins into epoxides. The mechanism consists of binding of the olefin to the metal to displace one of the peroxo-oxygen atoms, nucleophilic attack of the bound oxygen atom on the coordinated electron-deficient olefin, dissociation of the epoxide, and reaction of the remaining vanadium intermediate with

another alkyl peroxide. In this mechanism, there is a free coordination site on the metal and a labile ligand. The kinetic scheme is described by a Michaelis–Menten equation (*76*). It is tempting to speculate that such a mechanism occurs in vanadium bromoperoxidase; thus the reaction of bromide with the vanadium-H_2O_2 complex and transfer of an oxygen atom to the coordinated bromide occurs with formation and release of hypobromous acid. Of direct relevance is the observation by Secco (*78*) that at acid pH values vanadium(V) forms peroxo complexes with H_2O_2 and that these complexes are able to react with iodide in a nucleophilic reaction to form hypoiodite. In this mechanism no radicals are involved.

From comparison of antitumor activity and toxicity of hetero-ligand vanadium(V) complexes, Djordjevic and Wampler (*79*) arrived at the conclusion that the hetero-ligand is able to affect the redox potential of the V(V)/V(IV) couple in such a way that intramolecular electron transfer can occur within the V(V)-peroxoadduct. As a consequence, vanadium(V) is reduced to the IV state, and the peroxo group is oxidized to a superoxide radical. It is conceivable that such a species is present also during the reaction of vanadium bromoperoxidase with H_2O_2. However, there is no evidence for a radical type of reaction with bromoperoxidases.

It would be of considerable interest to see whether vanadium-peroxo complexes are also able to oxidize bromide and display kinetic behavior similar to that of the vanadium-containing bromoperoxidases. In this respect the complexes reported by Li et al. (*80*) may provide a useful contribution. Conversely, some attention should be paid to whether bromoperoxidases show specificity only toward bromide or iodide. These enzymes may perhaps be tuned to catalyze the oxidation and oxygenation of other nucleophiles.

Structural Properties of Vanadium Peroxo Complexes

It is very likely that during turnover of bromoperoxidase a peroxovanadium intermediate is formed and that peroxo (hetero-ligand) vanadate(V) adducts therefore represent a useful model for the bromoperoxidases. A great number of hetero-ligand peroxo complexes are known (*81–86*). X-ray structures have been determined (*74, 81–84, 86–92*) for several mono- and di-peroxo compounds, some of which are extremely stable. Vanadium(V)-peroxo complexes have sideways on peroxo ligation and can be regarded as seven-coordinate with a distorted pentagonal bipyramidal geometry. These complexes have V = O in an apical position with the peroxo group in the equatorial plane. Whether this also occurs in the vanadium bromoperoxidases is not yet known.

It is obvious that the proposed structure of the active site in the enzyme can only be confirmed when the three-dimensional structure of bromoperoxidase is available. Without this, it is not possible to obtain and interpret the details of the catalytic reaction. As such the crystallization of the bromoperoxidase reported recently is a first step in this direction (*93*).

IV. Vanadium in Mushrooms

The first report that vanadium occurs in fungal species dates back to 1931 when Ter Meulen (*94*) reported that fly agaric *Amanita muscaria* contained a high concentration of this element. Also in other Amanita species, such as *A. regalis* and *A. velatripes,* high amounts of vanadium (up to 400 ppm) were found (*95*). However, for a great number of European mushroom species much lower vanadium contents were reported (about 10 ppm) (*96*); vanadium in such high concentrations is restricted to a few species of the genus Amanita.

Apparently, vanadium is present in the (IV) state: The first EPR spectrum reported of an extract of the cap of the mushroom showed clearly an EPR signal characteristic of oxo-vanadium(IV) (*97*). Although the vanadium concentration in some mushrooms is low, it should be noted that they are similar to those reported for a number of seaweeds that may contain vanadium bromoperoxidases (*27*).

In 1972 Bayer and Kneifel isolated (*98*) a pale blue compound from *A. muscaria* containing vanadium, which they named amavadine. They proposed (*98, 99*) that it consisted of a complex of *N*-hydroxyimino-α,α'-dipropionic acid with VO^{2+} in a 2:1 ratio. From a comparison of the EPR spectra of segments of frozen mushrooms with those of vanadyl complexes of various amino acids, it was concluded (*100, 101*) that this proposal was not very likely. However, Krauss et al. (*102*) synthesized amavadine and compared its EPR properties with the complex extracted from the mushroom and concluded that it was the same. Others were unable to reproduce the synthesis (*103*), although models analogous to amavadine were reported. The synthesis of the ligand *N*-hydroxy-α,α'-iminodipropionic acid, and related compounds was, however, later confirmed (*104–106*). The stereochemistry and total synthesis of the vanadium compound of *A. muscaria* has now been elucidated (*107*).

The stability of the complexes of *N*-hydroxy-α,α'-iminodipropionic acid and some derivatives with alkaline earth, VO^{2+} and 3*d* divalent

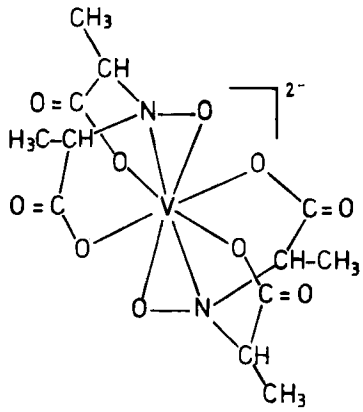


FIG. 3. The structure of amavadine as proposed by Bayer et al. (*109*).

cations have been studied in detail (*104–106, 108, 109*). Only with VO^{2+} are strong complexes formed and, to explain the very strong complex formation, it was pointed out that *N*-hydroxy groups participated in complex formation (*108, 109*). The original proposal (*98, 99, 107*) for the structure of amavadine was withdrawn, and the group arrived at a new model (Fig. 3) in which the V = O group is absent and in which vanadium(IV) is octacoordinated (*109*). In line with this, Carrondo et al. (*110*) recently succeeded in crystallizing the complex anion bis(*N*-hydroxy-iminodiacetate) vanadate(IV) which contains not a vanadyl but a vanadium(IV) complex anion in which the oxo group is removed and the hydroxy-imino group is ionized and coordinated to the metal. The special structure of vanadium(IV) eightfold coordinated to nitrogen and oxygen may cause the reported high stability. The complex lacks the band at 985 cm^{-1} in the IR spectrum reported for the natural product from *A. muscaria* (*99, 107*), which was previously assigned to the VO vibration. It would be most interesting to compare EPR spectra of this compound with that of natural amavadine.

Despite all these structural studies the physiological function of amavadine in mushrooms is still elusive. A suggestion has been made (*106*) that it may act as a cofactor with a protective oxidase or peroxidase function. On the other hand, the electrochemistry (*111*) of amavadine is such that it may have a role in electron-transfer reactions involving the vanadium(V)/(IV) redox couple.

V. Vanadium in Coal

Coal is known to be produced from vegetable material deposited in a swamp environment. The level of vanadium in coal is in general much lower than that seen in tar sands and oil shales, and less interest has been paid to the manner in which the element is held in coal. The average vanadium content of coal is 20 ppm (*71, 112*); this is low level probably explains why vanadium(IV) is not observed in a number of EPR studies on coal (*113–115*). However, much higher values of 1000 to 1800 ppm have also been reported (*111*) for certain types of coal. According to an early study by Triebs (*116*) on boghead coal, vanadium is present as the oxo-vanadium(IV) porphyrin. However, in a more recent X-ray absorption spectroscopy study on coal unusually rich in vanadium, no evidence was obtained for a porphyrin environment (*112*). It was found that vanadium exists in at least two environments in which it is coordinated to oxygen. The coal EXAFS could not be fitted with sulfur and the pre-edge position and intensity were in good agreement with V(IV) coordinated to oxygen with one short vanadyl bond. This coordination environment, however, may be peculiar to this type of coal.

VI. Vanadium in Tar Sands, Bitumen, Asphaltenes, and Crude Oils

Extraction of tar sands with benzene or toluene yields a bitumen fraction and a mineral fraction. Further treatment of the bitumen with heptane or *n*-pentane yields asphaltenes and petrolene, which are the insoluble and soluble fractions, respectively. Depending on the source, these fractions contain varying amounts of vanadium (*117, 118*).

The presence of this and other materials presents potential problems in industrial processing, and there is great interest in characterization of the chemical nature of the vanadium species present. EPR is most widely used in these studies since the metal is in the oxo-vanadium(IV) state. Optical spectroscopy in the visible region can also be used on extracts since oxo-vanadium(IV) porphyrins, which absorb at around 572 nm and 534 nm (*116, 119*), can readily be detected. However, it has been shown (*120–122*) that the total amount of vanadium present in crude oils and tar sand bitumen is higher than can be accounted for by the presence of oxo-vanadium(IV) porphyrins. It has therefore been suggested that the vanadyl may be bound to a range of different tetradentate ligands in crude oils (*120–123*).

A. Tar Sands and Asphaltenes

A careful analysis of the 35-GHz EPR spectra of a variety of asphaltenes was carried out by Malhotra and Buchmaster (*124*). They showed that two groups are observed typical of those for vanadyl square-planar complexes in the form of porphyrins, and that one group had EPR parameters identical to those for oxo-vanadium(IV) etioporphyrins. They also concluded that parameters such as g_0 and A_0 from model compounds cannot be used to predict coordination for various asphaltenes. Using EPR again and comparing the parameters with various vanadyl complexes, Shephard and Graham (*125*) concluded that the asphaltene fraction contained vanadyl etio-porphyrin. The results were confirmed (*126*) using electron nuclear double resonance (ENDOR) spectroscopy. Spectra were obtained from ^{14}H and ^{1}H nuclei and compared with those of vanadyl porphyrin complexes, and it was concluded that the porphyrin structure does indeed occur in the asphaltenes.

B. Oil Shale and Crude Oil

World-wide, vanadium is generally the most abundant trace metal in crude oil and shales (clay rock). There are, however, notable exceptions: Yugoslavian oil (*127*), for instance, contains a low concentration of the metal. Again, EPR is most widely used in studies of the way in which the metal is bound. Saracena et al. (*128*) have shown that almost all of the vanadium found in petroleum exists in oxidation state IV. Investigations of oils from different deposits (*121, 129, 130–132*) indicate that about 50% is in the form of porphyrins and that the remaining 50% consists of nonporphyrin vanadium(IV) components. This same conclusion has been reached more recently (*133*). Little is known about the chemical nature of the nonporphyrin complex, except that the vanadium is associated with compounds having a molecular weight of 9000 (*132*).

C. Biological Origin

The amazing richness of vanadium complexes in crude oil and shale raises the obvious questions of where these vanadium complexes come from and what their biogenic source is. It is generally believed that the vanadium porphyrins result from the incorporation of vanadium in porphyrin structures originating from chlorophyll. (The reader is re-

ferred to refs. *116* and *119* in which the geochemistry of porphyrins is treated in detail.) However, it cannot be excluded that the vanadium complexes come from a biogenic source. The discovery of vanadium bromoperoxidases, which are present in most brown seaweeds as well as some red seaweeds, suggests that the source of the vanadium may indeed be biological. In this respect the observation that oil shale deposits of marine origin contain substantial amounts of vanadium, whereas the abundance in shales of lacustrine origin is orders of magnitude less (*134, 135*), is of considerable interest.

VII. Vanadium in Tunicate Blood Cells

Tunicates, commonly called "sea squirts," are very successful marine organisms found in all the world's oceans. These animals (Phylum Chordata; Subphylum Urochordata; Class Ascidiacea) have a number of interesting features whose functions have eluded explanation for hundreds of years. Their blood, which possesses a diverse array of cells, conceals one such enigmatic trait. Blood cells of several ascidians accumulate astonishingly high concentrations of vanadium in lower oxidation states. Determining which specialized blood cells accumulate vanadium, the chemical forms of the accumulated vanadium, and the relation, if any, of associated blood pigments to the accumulated vanadium has been the subject of much research. This chapter therefore presents an assessment of the current understanding of vanadium in tunicate blood cells.

Previously, research on vanadium in tunicate blood cells was thought to be research on a single vanadium-accumulating blood cell called a vanadocyte. More recent research on vanadium in tunicate blood cells is converging on a different conclusion. No single cell should properly be regarded as a unique vanadium accumulator or vanadocyte. One type of research methodology responsible for this conclusion is directed at analyzing vanadium in blood cells by X-ray microprobe analysis. With this technique, cells in a whole blood preparation are examined individually and their elemental composition determined. Another approach is directed at gaining a better understanding of blood cell composition by chemical analysis. With this methodology, experimentation has progressed from whole blood extracts to experimentation with quite homogeneous populations of sorted blood cells. Results on intracellular vanadium distributions consistent with the microanalytical approach were eventually obtained using the chemical method.

Before describing these experiments in more detail, brief accounts of

ascidian blood-cell morphology and of accumulated vanadium characteristics are presented. Tunicate blood cell and vanadium function remain speculative and will not be covered here. A recent article (*136*) summarizes the status of evidence for a functional role of vanadium in tunicate blood cells.

A. Ascidian Blood Cells and Accumulated Vanadium

1. Blood Cell Classification

Identification of different blood cell types is normally carried out using light (optical) microscopy. Blood cells are collected and are either fixed or spread alive on glass slides and then examined microscopically. Many types of blood cells have been identified, and relative numbers and types of cells have been found to differ among tunicate species. Tunicate blood cells are fragile and readily change appearance. It is possible that some commonly accepted blood cell types may actually be artifactual.

Classification schemes have been devised that take into account the lability of tunicate blood cells (*137, 138*). In one scheme (*138*) four main categories of tunicate blood cell are recognized: stem cells, amoebocytes, vacuolated cells, and pigment cells (Fig. 4). Main categories are further subdivided; in *Ciona intestinalis,* for example, hyaline amoebocytes are numerous and comprise 30% of the total blood cell population (*139*).

Three vacuolar cells have been consistently associated with accumulated vanadium. These cells are spherical in overall shape and are 8–12 μ in diameter. The *signet ring cell* possesses one large spherical vacuole and is usually colorless or grey. The *morula cell* contains approximately 11–14 uniformly sized spherical vacuoles, giving the cell a mulberry-like appearance, and is colored yellow-green. This cell was originally termed the "vanadocyte" because it blackened on contact with easily reduced heavy metal stains such as osmium tetroxide, which was considered evidence for the presence of vanadium in reduced oxidation states. Morula cell vacuoles were thought to contain the accumulated vanadium and were termed "vanadophores" (*140*). Like the morula cell, the *compartment cell* is colored and possesses several vacuoles but, unlike morula vacuoles, they are neither spherical nor uniform in size.

2. Accumulated Vanadium

Improved analytical techniques have resulted in reliable data on accumulated vanadium concentration, oxidation state, and chemical environment. Using neutron activation analysis investigators exam-

Cell Type	OsO_4 / Neutral Red	Appearance
Lymphocyte	-/-	
Amoebocyte	-/red (vacuoles)	
Macrophage	-/-	
Nephrocyte	-/-	
Pigment Cell	-/-	
Signet Ring Cell	gray / light red	
Compartment Cell	gray / light red	
Morula Cell	black / deep red	

FIG. 4. Frequently cited ascidian blood cell types. The middle column refers to staining properties of each type of cell with the two reagents osmium tetroxide and the pH indicator neutral red. Free tunichrome would reduce osmium tetroxide, as would lower oxidation states of vanadium.

ined the concentrations of vanadium, iron, and manganese in the blood cells and tissues of 15 species of solitary ascidians (*141*). Vanadium was detectable in all species examined; however, the vanadium content of species in suborder Phlebobranchia was higher than either iron or manganese. Of all the tissues studied blood contained the highest concentrations of vanadium due to accumulation of the element in blood cells; plasma contained relatively little vanadium. The highest value recorded in this study was 21 μV/mg dry weight in blood corpuscles of *Ascidia ahodori*. Vanadium contents of species in suborder Stolidobranchia were less than those of either iron (highest) or manganese. Significantly, the iron and manganese contents of both suborders were roughly the same, but the vanadium contents were considerably higher for Phlebobranch species.

It should also be noted that ova of ascidians, unusual in containing somatic cells from the parent organism, are quite high in vanadium

content. The somatic cells, called "test cells," may be derived from parental blood cells (*142*).

Vanadium is present in sea water as the vanadate(V) monomeric anion. In the process of accumulation, it is reduced to a mixture of vanadium(III) and (IV). One oxidation state predominates, and this fact has been used as a biochemical criterion of taxonomy. Species of suborder Aplousobranchia accumulate vanadium as vanadium(IV) and those of suborder Phlebobranchia predominantly as vanadium(III); the Stolidobranchia do not accumulate significant amounts of vanadium (*143*).

The EPR technique has been used extensively to identify and characterize oxo-vanadium(IV) (i.e., vanadyl) in tunicate blood cells. Although EPR spectra are clearly diagnostic of vanadyl, different species yield different values of vanadyl EPR parameters (A and g values). Different values of EPR parameters have been reported for species such as *A. ceratodes* (*144, 145*), *Leptoclinides lissus* and *Phallusia julinea* (*143*). Vanadyl EPR A_0 values of the blood cells of *A. ahodori* collected from different locations in the waters off Japan have been reported to differ from one another by approximately 10% (1.06×10^{-2} cm^{-1} vs. 0.95×10^{-2} cm^{-1}), an amount about as much as interspecies differences (*146*). This result has been questioned, however, in the context of an extensive study of both Aplousobranch and Phlebobranch species utilizing several different methods to detect intracellular vanadium (*147*).

Hawkins and co-workers find significant vanadium(IV) in all vanadium-containing Aplousobranchs and little or no vanadium(IV) in Phlebobranchs. Vanadium(III)-accumulating specimens (Phlebobranchs) were then exposed to the atmosphere, which facilitates vanadium oxidation, and the EPR spectra were rerun. Two types of EPR resonance (I and II) could then be identified in these "oxidized" Phlebobranch specimens. Most of the vanadium(IV)-accumulating Aplousobranchs exhibited Type II resonance, and most vanadium(III)-accumulating Phlebobranchs exhibited Type I resonance, albeit some specimens required oxidation before a significant vanadium (IV) EPR signal could be obtained. Both Type I and II resonances most closely resemble model systems based on vanadyl coordinated to organic ligands. This analysis requires data-fitting by simulation. The *A. ahodori* data, which should belong to Type I, were not so analyzed and this fact may account for the observed differences formerly ascribed to different collection sites. EPR A and g values are expected to differ when the structure and type of bonding in the vanadyl ion's inner coordination sphere differ. However, while the last word on EPR analy-

sis of tunicate blood cells is yet to be written, it is clear that any complete description of accumulated vanadium must accommodate several different types of intracellular environments.

Two techniques capable of positive identification of both the vanadyl and the vanadium(III) oxidation states are extended X-ray absorption spectroscopy (EXAS) and magnetic susceptibility. EXAS experiments on *A. ceratodes* yield approximately 95% V(III) and 5% V(IV) (*148*). A similar distribution, 90% V(III) and 10% V(IV), was found for *A. nigra* using a superconducting quantum interference device to measure magnetic susceptibility (*149*).

Elements other than vanadium are accessible to the EXAS technique. Thus, abundant sulfur detected in tunicate blood cells has been identified as sulfate and sulfonate with EXAS (*150*). This result confirmed a similar finding carried out with chromatographic techniques (*151*). With this background, let us now consider the X-ray microprobe analyses of tunicate blood cells.

B. X-ray Microanalysis of Tunicate Blood Cells

Analysis of X-rays produced by the minute beam of an electron microscope probe provides elemental analysis of cells and subcellular components. Early studies utilizing this technique with several ascidians indicated relatively little vanadium in morula cells and significantly more vanadium in amoebocytes, signet ring cells, and compartment cells (*152*). Moreover, the vanadium was found mainly on membranes and granules of vacuoles rather than within the vacuole or coincident with the electron-dense region of an electron micrograph. These studies were not free from problems, however. Identification of cell types was made difficult by damage that could also cause loss of material from the cells. Improvements in the technique followed; for example, freezing cells in liquid propane and then sectioning the frozen pellet. Such studies on *P. mammillata* confirmed the relatively low concentration of vanadium in morula cells of this species compared with other cell types (*153*).

Blood cells of *C. intestinalis* showed low and variable levels of vanadium in morula cells (*154*). In this species the primary vanadium storage site was determined to be the granular amoebocyte. It was first concluded that significant vanadium is present in the morula cells of the species *A. mentula* and *Ascidiella aspersa,* but "the various cell types were not distinguishable" (*155*). When the same two species were reexamined with improved fixation methods it was concluded that sig-

nificant amounts of vanadium occurred in three cell types: morula, signet ring, and granular amoebocytes (*156*).

The species *A. mentula* was the subject of a more accurate electron probe X-ray microanalytical study which concentrated solely on vanadium, sulfur, and bromine in morula cells (*157*). In this species the vanadium content of morula cells is quite high. Vanadium concentrations in fixed *A. mentula* morula cells would correspond to a concentration of 0.5–0.65 M *in vivo*. The concentration of sulfur depended strongly on the method of fixation but was high and could be equimolar with vanadium. The authors concluded that there might be at least one sulfur-containing nonwater complexing site in the vanadium(III) inner coordination shell. The finding of bromine in the blood cells is interesting in view of the foregoing discussion of the biological activity of halogen metabolites in marine organisms, but the X-ray microprobe determination of this element is highly subject to error and no confidence could be placed in the accuracy of the data.

Staining cells of Phlebobranch species with complexing ligands such as 2,2′-bipyridine revealed the presence of vanadium(III) in compartment, signet ring, and morula cells (*147*). Often, however, pigmented morula cells did not stain positively with such ligands, even though electron microprobe analysis gave positive evidence for the presence of vanadium(III), for example, in the morula cells of the species *Ecteinascidia nexus* (*147*). Lack of staining may be due to the inability of the incoming ligand to replace the endogenous ligand, or it may be due to the insoluble nature of accumulated vanadium. Alternatively, the pigment may react with the stain and diminish its ability to chelate metal ions.

It seems fair to conclude from these studies that several different blood cells accumulate vanadium. Whether this result is due to the occurrence of vanadium in transitory cells representing morphologically different stages during blood cell development or to the distribution of vanadium in several distinctly different cell types will not be settled until more is known about the sequence of blood cell development. Assignment of a unique position to the morula cell in vanadium accumulation no longer seems appropriate. In *P. mammillata* and *A. nigra* this blood cell contains relatively little vanadium, in *A. mentula*, *A. ceratodes*, and *E. nexa* it contains significant amounts of vanadium. Among tunicate blood cells, however, the morula is the predominant pigmented cell. The chromophore producing morula cell coloration and its possible relation to vanadium accumulation is considered next.

C. Tunichromes

1. Isolation and Purification of Tunichromes

The yellow-green coloration of morula cells was originally ascribed either to vanadium complexes within these cells or to a macromolecular cell constituent (see *158* for early literature). Careful spectroscopic studies showed that the green color of *A. ceratodes* blood cells was not due to inorganic complexes of vanadium(III) or (IV) (*159*). Chromatographic separation showed that the yellow-green color of *A. nigra* blood cells was due to a low molecular weight organic compound that retained cell coloration after removal of intracellular vanadium (*160*). This compound was named tunichrome by its discoverers. Subsequently, a scheme for the isolation and characterization of what turned out to be several homologous tunichrome compounds was developed (*161, 162*).

The problem of isolating pure blood pigments from tunicates is very difficult owing to the extreme air-sensitivity and reactivity of the native compounds. Several steps and techniques proved to be necessary to produce a few milligrams of purified material. Structure elucidation following isolation of the purified pigment is also a complex procedure. The main steps leading to structure determination are as follows.

1. *Lyophilization,* which requires great care to produce a free-flowing green powder uncontaminated by decomposition and oxidation products.
2. *Extraction chromatography,* through which cellular contaminants such as carotenoids are removed and a concentrated crude extract of a mixture of tunichromes is prepared.
3. *Centrifugal counter-current chromatography,* which provides a relatively rapid, nondestructive technique for separating individual tunichromes. Great losses are encountered during this phase of the procedure.
4. *Preparative high-pressure liquid chromatography,* which is the only technique currently available that has sufficiently high resolving power to separate a pair of tunichromes.

The resulting purified material is more stable than the impure samples and could be characterized by a variety of spectroscopic and chemical conversion techniques. In addition, an assay of crude blood-cell extract for the presence of tunichrome was developed (*162*). Tunichrome blood pigments consist of a number of closely related polyphenolic compounds with a central triglycyl unit (Fig. 5). A tunichrome (designated An-1) isolated from the species *A. nigra,* for example, of

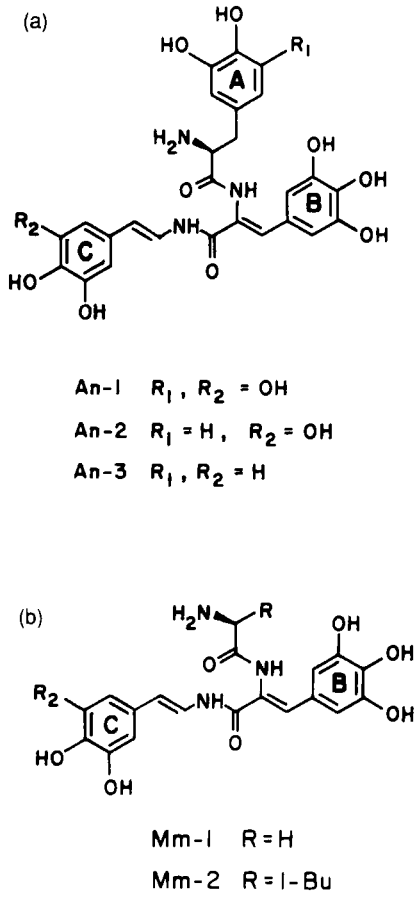


FIG. 5. The structures of tunichromes (blood pigments) from *A. nigra* consist of a number of closely related compounds (a) designated TC An-*i* (*i* = 1, 2, . . .); those obtained from *M. manhattensis* (b) are designated TC Mm-*i*.

formula $C_{26}H_{25}N_3O_{11}$ and molecular weight 555, consists of three trihydroxyphenylalanine groups joined by peptide bonds. Tunichromes from *Molgula manhattensis* contain only two phenolic rings, one of them having been replaced by glycine.

Underivatized (±)-tunichrome An-1 was synthesized recently on a semi-preparative scale (*163*). Its availability will help to clarify the biological role of tunichromes, including their interactions with vanadium.

2. *Tunichrome–Vanadium Interactions*

Tunichromes have metal reducing and chelating power; therefore, it is natural to speculate whether this compound participates in the accumulation of vanadium. In vivo evidence for such a function has been found in connection with tunichrome isolation (*162*). In addition to free tunichrome, Sephadex LH-20 chromatography of lyophilized *A. nigra* blood cells yields a green fraction containing vanadium bound to tunichrome. In vitro evidence consists of compounds formed when dilute aqueous solutions of vanadium(V) or (IV) are added to aqueous acidified solutions of tunichrome. Starting with vanadyl a crystalline dimer of V(III) bridged by pyrogallol has been prepared (*164*). Since early attempts to reduce V(V) to V(III) by crude aqueous tunichrome extracts yielded only V(IV) (*165*), success in preparing the pyrogallol dimer of V(III) suggests that complexation to tunichrome may occur in a hydrophobic blood-cell environment. Although indicative of a role for tunichrome in the assimilation of vanadium, this evidence is not proof of such a relation. A reducing sugar has also been proposed as a vanadium binding substance (*166*).

D. Vanadium Distribution and Stabilization

1. *Vanadium Distribution in Tunicate Blood Cells*

Cell lysis, which occurs in the course of chemical analysis of whole blood, allows the contents of different cells and separated components of the same cell to mix and react. To avoid the complications resulting from this procedure it should be possible to separate the blood cells prior to chemical analysis. The first such attempt was applied to *A. nigra* blood cells using Lymphoprep density fraction and atomic emission vanadium analysis (*167*). Although homogeneous blood cell populations were not achieved in this study, it was clear that vanadium was distributed in several blood cell types. Better separation and resolution of cell types was obtained with Ficoll density gradients (*168*). It was shown that the signet ring cells of *A. ahodori* have a much higher vanadium content than the morula cells of this species of ascidian. The best separation of ascidian blood cells to date has been achieved with flow cytometry (*169*). A fraction of approximately 99% signet ring cells could be achieved using laser light scattering and fluorescence as cell sorting criteria. Two species were studied and both vanadium and tunichrome content measured in the sorted blood cell populations. The majority of vanadium in *A. nigra* blood cells occurs in signet ring cells with lesser amounts in the morula cells; in *A. ceratodes,* significant amounts of vanadium are found in both signet ring and morula cell

fractions. For both species *free* tunichrome occurs mainly in morula cells.

These results support the electron X-ray microprobe studies. The term "vanadocyte" as applied to a single cell appears to be a misnomer as vanadium is distributed in more than a single cell in at least five species of the genus *Ascidia* and one of *Ciona*. Although certainly a more accurate description of vanadium histochemistry, this finding leaves unanswered the question of how vanadium is accumulated in such a low oxidation state.

2. *Stabilization of Intracellular Vanadium(III) and (IV)*

The occurrence of vanadium in the lower oxidation states, which as the simple aqua ions undergo acid dissociation above pH 3 [if present as V(III)] and pH 6 [in the case of oxo-V(IV)], along with the high sulfur content of ascidian blood and the low pH that results when ascidian blood cells are ruptured in distilled water has led to the belief that intact vanadium-containing tunicate blood cells are acidic (*145*). Other lines of evidence, including vital staining and ^{31}P NMR (*144, 170*), and the distribution of ^{14}C methyl amine (*171*), suggest a neutral or mildly acidic intracellular pH. This conflict may arise from how the vanadium-containing milieu is viewed and may not be due to fundamental errors in methodology.

The X-ray microanalytical studies suggest that accumulated vanadium is not found in aqueous intracellular volumes such as vacuoles but occurs mainly in hydrophobic granules and membranes. Chelation by a mixture of ligands including water, sulfate, and tunichrome could have two effects on the lower oxidation states of vanadium, namely, to decrease the reduction potential and increase the stabilization at physiological pH. The EPR line-width studies (*145*), carried out on whole blood samples and based on aqua vanadyl models, may simply be inappropriate or applicable to only a fraction of the accumulated vanadium.

Transport of vanadate(V) into tunicate blood cells is nonenzymic and occurs through anionic channels (*172*), which is consistent with a trapping mechanism of vanadium accumulation (*162*) in which cationic reduced vanadium cannot leave the cell. Chelation by a variety of reducing ligands such as tunichromes or catechol-containing compounds similar to those found in marine organisms (*173*) would augment this mechanism. However, there is also evidence that vanadium may be complexed by proteins in the plasma (*174*), which implies that reduction may have already occurred prior to transport into a blood cell. Clearly, considerable experimental work with this fascinating biometallic system remains to be done before the intracellular environment and function of vanadium in tunicates can be understood.

VIII. Key Words and Abbreviations

Key Words:
vanadium
bromoperoxidase
catalysis
mushrooms
vanadium-porphyrins
oil
tunicates

Abbreviations:
EPR, electron paramagnetic resonance
EXAS, Extended X-ray absorption spectroscopy
EXAFS, Extended X-ray absorption fine structure
ESEEM, Electron Spin Echo Envelope Modulation Spectroscopy
Tris, (tris hydroxymethyl)amino methane

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