Proc. Indian natn Sci Acad. **B69** No. 4 pp 649-672 (2003)

Current Opinion

The Emerging Redox Profile of Vanadium

T RAMASARMA*
Solid State & Structural Chemistry Unit and Department of Biochemistry
Indian Institute of Science, Bangalore 560 012

(Received on 4 April 2003; Accepted after revision on 13 June 2003)

Multiple biological roles of vanadium include essentiality in traces, therapeutic effects in small doses and toxicity in excess. Revival of interest in biochemistry of vanadium is triggered by the discoveries of its unusual inhibition of many phosphatases, its insulin mimic actions, its selective oxidation of NADH and bromide and its natural occurrence in some enzyme proteins. It occurs as monomeric (V_1) and oligomeric (V_2, V_4, V_{10}) forms. Decavanadate (V_{10}) has a compact cage-like structure and its V^V is the preferred form for reduction by chemical and enzyme reactions. Only two of its V-atoms convert to a vanadyl (V^{IV}) form that has several fold higher absorbance compared to the monomer. And this reduced form plays a crucial role in forming highly reactive μ-peroxo complex in NADH oxidation. The $^{\bullet}$OV-type radicals formed during redox reactions of vanadyl and diperoxovanadate (DPV) at physiological *p*H act as selective oxidants of compounds such as NADH, ethanol, benzoate and bromide. Pathways and cycles of peroxovanadate intermediates operating for these oxidations are constructed to fit the available information. The catalase-stable DPV also modulates activities of many enzymes, particularly those of signal transduction (protein kinases and phospholipases C/D). For the first time an integrated scheme of vanadate pathways and peroxovanadate cycles are presented.

Key Words: Vanadium redox, Decavanadate reduction, Diperoxovanandate, NADH oxidation, Bromoperoxidation mimic, $^{\bullet}$OV-type radical, Enzyme modulation by peroxovanadate, Vanadate-peroxovanadate cycles

Introduction

Vanadium, the 21st most abundant element in the earth's crust, occurs in biological materials only in trace amounts. Some accessory foods such as black pepper, tealeaf, cocoa powder and some mushrooms contain relatively high amounts of vanadium. Vanadium is acclaimed as "*panacee universelle*" a century ago in recognition of its pharmacological benefits in treatment of diseases as diverse as anemia, tuberculosis, syphilis and diabetes. Vanadate has a place in homeopathic materia medica for treatment of wasting diseases (2 mg/day). A tonic (neogadine) containing metavanadate (44 μg/ml) is marketed in India. Vanadyl sulfate is touted as sports medicine for muscle build-up, unmindful of the toxicity, based upon slender scientific support that it might be anabolic (Moore & Friedl 1992). Excess intake is becoming unavoidable in view of inhalation of dust and smoke arising out of burning vanadium-rich fossil fuels in industries and automobiles. Chronic exposure gives a range of toxic responses such as anaemia, leucopenia, renal and bones damage, diarrhoea, sperm loss, bronchopneumonia, asthma, nausea, vomiting, bradycardia and coronary insufficiency (Vouk 1979, Domingo et al. 1991). The multiple biological roles of vanadium include its essentiality in traces, therapeutic effects in pharmacological doses and toxicity in excess.

Revival of Interest in Vanadium Biochemistry

Simple discoveries and timely reviews around 1980 (table 1) had dramatically enhanced the awareness of importance of vanadium in biology. The discovery that vanadium present as a trace contaminant in horse muscle derived ATP was enough to block sodium pump activity triggered studies on biochemical action of vanadium (Cantley Jr. 1977).

* *Corresponding address*: *Email:*trs@biochem.iisc.ernet.in, *Tel.* (080) 2932309, *Fax:* (080) 2931310

Table 1. *Resurgence of interest in biological actions of vanadium*

Reaction/Parameter	Vanadium	Effect/Locale	Reference
Na,K-ATPase	vanadate	inhibition	Cantley Jr. et al.(1977)
Insulin-mimic	vanadate	blood glucose$^-$	Dubyak & Kleinzellar (1980)
Insulin-mimic	vanadyl	blood glucose$^-$	Schector & Karlish (1980)
Noradrenaline-mimic	vanadate	arterial contraction	Ozaki & Urokowa (1980)
NADH-V reductase	vanadate	plasma membrane	Erdmann et al. (1979)
NADH-O_2 oxidation	polyvanadate	plasma membrane	Ramasarma et al. (1981)
Nitrogenase mutant	vanadate	*A.vinelandii*	Robson et al. (1986)
Bromoperoxidase	vanadate	marine alga	de Boer et al. (1986)

The article entitled "Vanadium, a new tool for biologists" by Simon (1979) served as a wake-up call. Giving a list of effects known by then by different forms of vanadium, Macara (1980) described it as "an element in search of a role". By that time, vanadate was found to inhibit most phosphatases (with the exception of F_1-ATPase) and thereby enhanced the effectiveness of phosphate esters including many protein-phosphates, the products of regulatory protein kinases.

The discoveries of receptor-independent insulin-mimic action of vanadate (V^V) (Dubyak & Kleinzeller 1980), and of vanadyl (V^{IV}) (Shector & Karlish 1980) created deserved excitement. About the same time, vanadate was reported to mimic noradrenaline activity in contracting pulmonary artery (Ozaki & Urokawa 1980) and in increasing contraction force of cardiac muscle (Hackbrath et al. 1980), however these findings received less attention.

Stimulation of NADH oxidation by plasma membranes on addition of vanadate highlighted the redox basis of its action. This activity, based on disappearance of NADH in the presence of vanadate using calf cardiac muscle membranes as the enzyme source, discovered by Erdmann et al. (1979) was rightly considered a vanadate reductase. This must be limited to the reduction segment of added vanadate and its non-enzymic reaction had a *p*H optimum around 7.0 (Vyskocil et al. 1980). In the expanded work described by Ramasarma et al (1981), NADH oxidation exhibited an explosive enhancement of rates at acidic *p*H, sans optimum. For the first time, the accompanying consumption of oxygen with stoichiometry of NADH : O_2 of 1:1 was also demonstrated. Paradoxically, this H_2O_2-generating oxygen-consuming reaction was found to be sensitive to superoxide dismutase (SOD), the enzyme known to promote dismutation of superoxide to H_2O_2. This unexpected and unusual effect spurred research on the redox profile of vanadium.

In the first detailed review on physiological and biochemical effects of vanadium compounds, Ramasarma and Crane (1981) raised the query: "Does vanadium have a role in cellular regulation?" Additional information that accumulated since then vindicated the optimism regarding the versatility of vanadium. Besides interfering with phosphate ester hydrolysis, many other reactions of vanadium compounds have been discovered.

Discoveries of proteins containing bound vanadium as a native constituent and essential for the activity of a mutant nitrogenase in *Azotobacter* (Robson et al. 1986) and of a bromoperoxidase in a marine alga (de Boer et al. 1986) left no doubt on biological relevance of vanadium. The title "A role for vanadium at last" of the review by Commack (1986) of these findings expressed relief. Interestingly both these enzymes depend on redox reactions. A profile is presented in this note on some redox reactions of vanadium and their biochemical implication.

Complicated, yet Useful Reactions of Vanadium

Due to its high toxicity, scant occurrence, and "exceptionally complicated chemistry in solution, multiple oxidation states, hydrolysis and polymerization" (Macara 1980), interest on vanadium studies remained at a low profile. Orthovanadate and its oligomers show effects by competition with substrates and by some structural interactions with enzyme proteins (Crans 1994). A variety of naturally occurring organic compounds such as carboxylates, catechols,

phenolics, nucleoside derivatives, amines, amino acids, peptides, and proteins form complexes with vanadate and peroxovanadates (Crans 1995). Commonly used organic buffers and EDTA form complexes with vanadium compounds and these are often inhibitory. Vanadium uses this very complexity to accomplish unusual reactions. Speciation of vanadate compounds, resembling that of phosphate, depends on concentration and *p*H. At low concentrations (< 0.1 mM) monomeric vanadium predominates with dioxovanadium (OVO^+) at *p*H < 3.5 and orthovanadate (VO_4) with decreasing protonation as *p*H increased to *p*H 9.0. Greatly influenced by *p*H and high concentrations, the species present are OVO^+ at *p*H < 3.0, the decamer ($V_{10}O_{28}^{6-}$) with decreasing protonation between *p*H 3.0 and 7.0 and the tetramer ($V_4O_{12}^{4-}$) at *p*H 6.0 and above. At physiological *p*H, a mixture of monomers and oligomers is present in aqueous solutions. Their characteristic peaks in ^{51}V-NMR spectra are most helpful in identifying the species present by comparing with the standards provided by Howarth andHunt (1979), Harrison and Howarth (1985) and Jaswal and Tracey (1991) (table 2). In our studies, especially in neutral phosphate buffer and varying vanadate concentrations, small variations were found in the values of these peaks. Study of ^{51}V-NMR spectra provided invaluable tool in identifying vanadium intermediates in the reactions of vanadate and vanadyl with H_2O_2.

Decavanadate (V_{10}) and Its Formation

It is common to use ammonium metavanadate (NH_4VO_3) or dissolve vanadium pentoxide (V_2O_5) in alkali to obtain vanadate solutions. Solutions of metavanadate on storage or on acidification turned yellow. The alkali extract of V_2O_5 invariably yielded a yellow solution. This colour indicates presence of decamer, the active form in many cases. The standard procedure recommended for its depolymerization was to heat the solution. Indeed a lack of appreciation for the presence of undefined amounts of V_{10} in solutions led to variable results and controversies [see discussions in Kalyani and Ramasarma (1992) and Aparna Rao and Ramasarma (2000)].

For the first time, we characterized the products present in such solutions, progressively changing

Table 2. *Species, formulae, ^{51}V-NMR peaks (ppm) of some vanadium compounds*

Number of Peroxo Groups	Species	Chemical Shift (δ) in ^{51}V-NMR Peaks (ppm)		
		Howarth & Hunt (1979)	Harrison & Howarth (1985)	Jaswal & Tracey (1991)
Nil	OVO^+		-546	
	VO_4^{3-}	-545	-541	
	HVO_4^{2-} (V_1)	-534	-539	-536
	$H_2VO_4^-$ (V_1)	-574	-564	-566
	$HO(VO_3)_2^{3-}$ (V_2)	-562		-574
	V_4 cyclic			-578
	V_4 linear or V_5			-582
One	$VO(O_2)^+$	-543	-549	
	$VO(OH)_2(O_2)^-$	-621	-602	
	$VO_2(OH)(O_2)^-$	-623	-628	-625
	$[VO_2(O_2)]_2O^{4-}$ (dimer)		-636	
Two	$VO_2(O_2)_2^{3-}$	-760	-769	
	$V(OH)_2(O_2)_2^-$	-696	-700	-686
	$VO(OH)(O_2)_2^{2-}$	-767	-771	-765
	$[VO(O_2)_2]_2OH^{3-}$ (dimer)	-757	-767	-758
	$[V(OH)_2(O_2)_2]_2O$ (dimer)	-650		
Three	$VO_2(O_2)_3^{3-}$	-845	-830	
	$VOH(O_2)_3^{2-}$	-733	-737	

from V_1, V_2 and V_4 to V_{10} by ^{51}V-NMR spectra (Kalyani & Ramasarma 1992). Prolonged extraction of solid V_2O_5 with dilute NaOH gave initially a light yellow solution that showed a major peak at -576 ppm (V_4), a small one at -584 ppm (V_5?) and a trace of triple peaks corresponding to V_{10}. Subsequent slow extraction contributed acid equivalents and facilitated progressive polymerization to V_{10}. At periods of 48hr and above, the solution exclusively contained V_{10} (-516 ppm, -500 ppm and -424 ppm) (figure 1, top panel). Decavanadate can be obtained as yellow-orange crystals ($Na_6V_{10}O_{28}$, 18 H_2O) by layering cold ethanol or acetone on this extract and leaving for a few days in the cold. Availability of such pure crystalline form V_{10} greatly helped in our studies.

An aqueous solution of ammonium metavanadate (30 mM) at *p*H 7.0 showed a major peak at -576 ppm (cyclic-V_4) accompanied by traces of -558 ppm (V_1), -572 ppm (V_2) and -584 ppm (V_5, originally thought to be linear V_4) but none of V_{10}. Kept at *p*H 5.0 for 4-week incubation at room temperature such a colourless solution of metavanadate turned yellow. All vanadium therein had been converted to V_{10} (-516 ppm, -500 ppm and -424 ppm) (figure 1, middle panel).

Oligomerization, a characteristic of vanadate, occurs with ease even in aqueous solutions and was accelerated on adding KBr, also known to reduce V^V to V^{IV} (Aparna Rao et al. 1996, Venkataraman et al. 1997). Significant increase in V_{10} content (-515 ppm, -498 ppm and -426 ppm) was found in a solution of metavanadate at *p*H 5.5 after 10 min incubation with KBr. This reaction was accompanied by formation of V_5 (-584 ppm), implicating it as a possible intermediate (figure 1, bottom panel). From these experiments, it appears that V_4 present in solutions of vanadate in acid medium tends to be converted to V_5 and then to V_{10}. Assistance of bromide in this process indicated that one or more of vanadium atoms in the reduced form might aid the joining together of the two identical halves of V_5.

The polymerized product, decavanadate (V_{10}), acquires a cage-like structure, a yellow-orange color and two pairs of rare triply shared oxygen atoms between vanadium atoms 3.12 Å apart bridging the two segments of the oligomer (Debaerdemaeker et al. 1982). The crystal structure of decavanadate

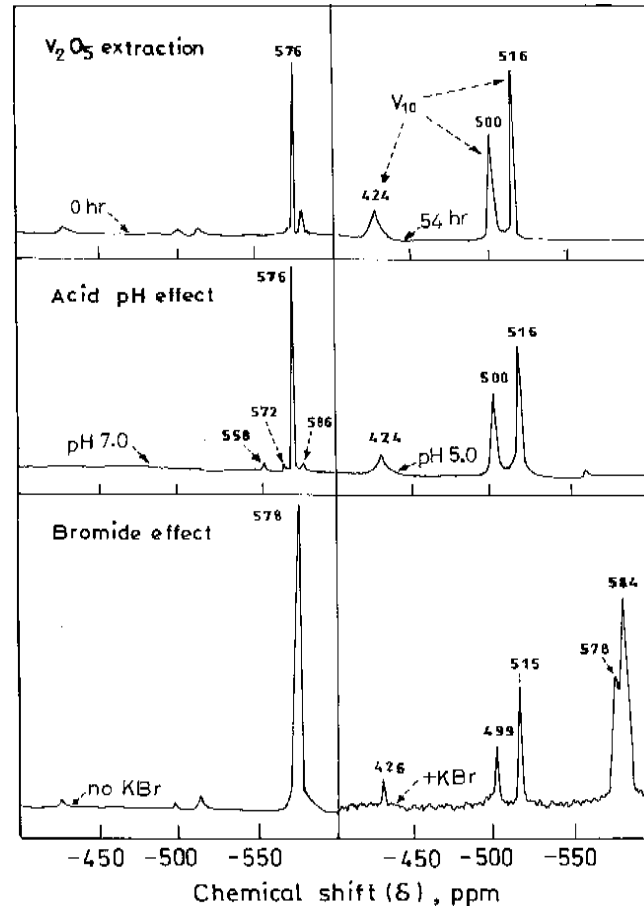

Figure 1. ^{51}V-NMR spectra indicating the formation of decavanadate from vanadium compounds. Top: solid V_2O_5 was extracted with 0.3M NaOH and clear supernatants were analyzed at the stated hours. Middle: metavanadate (30 mM) was adjusted to stated *p*H and kept at room temperature for 4 weeks. Bottom: metavanadate (10 mM) in phosphate buffer (50 mM, *p*H 5.5) without and with KBr (2M) incubated for 10 min (adapted from Venkataraman et al. 1997).

($V_{10}O_{28}^{6-}$) anion showed it to be a highly condensed system of ten distorted octahedra symmetrically arranged in two segments around a center, shown by a dot (figure 2). The "454 electrons are trapped inside a finite volume enclosed by the minimal surface" (Gadre et al. 1992). The 28 O-atoms (identified by subscript numbers on the right) coordinate with 1, 2, 3 or 6 V-atoms (identified by superscript numbers on the right) are classified into four types, I-IV, respectively and play a role in maintaining the structure (Venkataraman et al. 1997). Type I and type II of these are present in V_4 and V_5, and type III and type IV arise only on oligomerization to V_{10}. Two unusual buried oxygen atoms (O_1 and O_{1B}) have six V-atoms surrounding each at a weakly bonding distance of 2.75 Å.

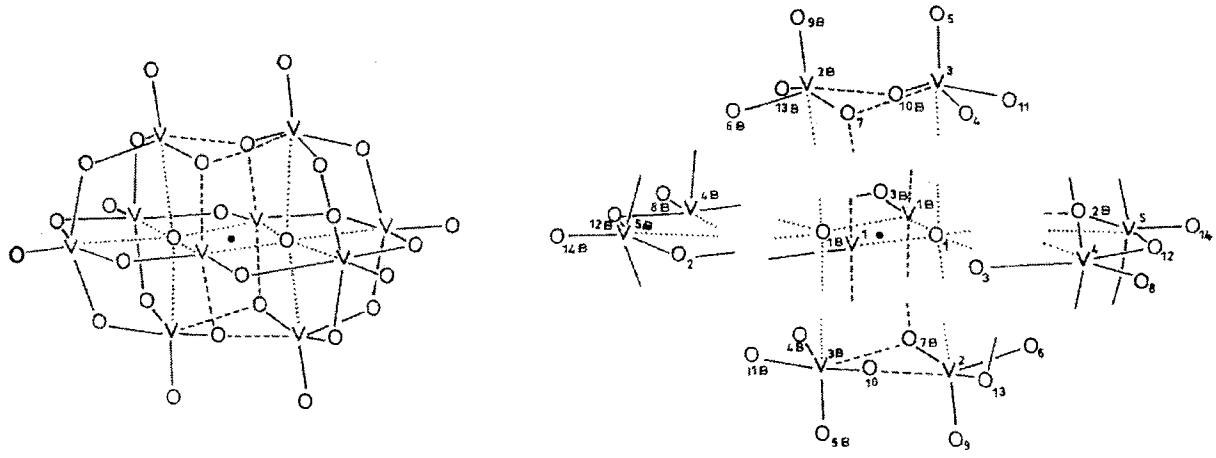

Figure 2. Structural features of decavanadate (V_{10}). Computer graphic model of V_{10} using crystallographic parameters of Debaerdemaeker et al. (1965) is shown on the left. The atoms are numbered $V^{1\text{-}5}$ and $O_{1\text{-}14}$ for one half and B series for the other half, related to each other by a centre of symmetry shown by a dot. Notice oxygen atoms, two each at the top and bottom, which are triply shared indicated by dotted lines. On the right is a blow up of the molecule indicating three types of V-atoms, 2 at the centre, 4 at the top and bottom and 4 at the left and right.

O-atoms (7,10, 7B, 10B) in the middle that bridge the two segments of the molecule by undergoing unusual coordination with three V-atoms each. In the study of the basicity of the oxygen atoms, Gadre et al. (1992) found that the deepest molecular electrostatic potential (MESP) minimum occurs in these four O-atoms, thereby making them most negative (partially reduced?). The cage-structure is maintained by six O-atoms (1,7,10 and their B series) and it will be of interest to see if their unusual interactions play any role in the functions of V_{10}.

The 2:2:1 pattern of the NMR spectrum (triple peaks between -520 ppm and -420 ppm) indicates that three types of V-atoms are present in the ratio of 4:4:2 in V_{10}. An attempt is made here to identify these in the blow-up shown in figure 3 (right panel). V^1 and V^{1B}, associated with the loosely held O_1 and O_{1B} in the center, is the only pair of V-atoms different from the rest. They automatically claim the -420 peak. The two pairs of V^{2B} and V^3 on the top and V^{3B} and V^2 at the bottom that seem to join the two parts through the triply-shared O-atoms are another set of four V-atoms that differ from the others; the middle (-500 ppm) peak probably arose out of these. The rest of four V-atoms, V^{5B} and V^{4B} on the left and V^4 and V^5 on the right, seem related to V_2-dimer and may be assigned the other peak at -520 ppm.

Reduction of Vanadate (V^V)

Biological vanadium exists in three valency states, 5+ (V^V), 4+ (V^{IV}) and 3+ (V^{III}). The oxidized anion, V^V, is reduced to the blue cation, V^{IV}, by cellular reductants such as ascorbate (Kustin & Toppen 1973), glutathione (Mecara et al. 1980), noradrenaline (Adam-vizi et al. 1981) and sugars (Liochev & Fridovich 1987). Clarity is yet to emerge on how V^{III} is formed.

An aqueous solution of V^{IV} (vanadyl sulfate, $VOSO_4$) is blue in color and has a broad absorbance peak in the visible spectrum around 750 nm) (figure 3, left panel). In the presence of phosphate buffer (20 mM, *p*H 7.0) absorbance at 750 nm decreases considerably as the peak shifts to about 600 nm (Ravishankar & Ramasarma 1993). Turbidity at 1 mM concentration of vanadyl in phosphate buffer was small enough to allow recording of the spectrum at high

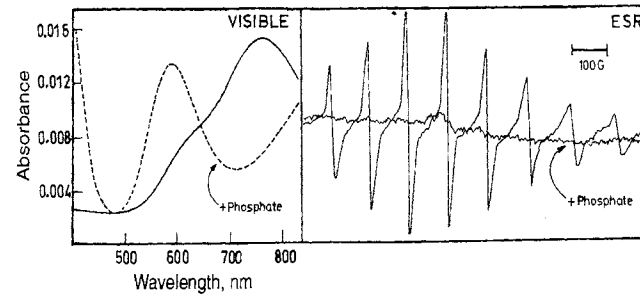

Figure 3. Visible and ESR spectra of vanadyl sulfate in the absence and the presence of phosphate buffer (20 mM, *p*H 7.0). Note the shift of the peak in the visible (vanadyl, 1 mM) and disappearance of signals in ESR (vanadyl, 3 mM) in presence of phosphate buffer (broken lines) (adapted from Ravishankar & Ramasarma 1993)

to allow recording of the spectrum at high sensitivity. The peak of the reduced form varies in the range of 600-750 nm depending on the reducing system, *p*H and the medium.

Most experiments on chemical reduction of vanadate were done in highly acidic media. As discussed above, V_{10} would invariably be present under these conditions. V_{10} was found to be more active for reduction of V^{V} (Ramasarma et al. 1981, Patole et al. 1987). A solution of metavanadate in 1.0 M $HClO_4$ is reduced to blue-colored vanadyl (V^{IV}) by ascorbic acid (Kustin & Topper 1973) and V_{10}, present in the reaction mixture, is likely to be the species reduced.

A typical experiment of reduction of aqueous solutions of metavanadate and crystalline decavanadate by ascorbate is shown in figure 4 (lines i, ii). The samples used were examined by ^{51}V-NMR spectra and found to be pure - metavanadate having no V_{10} and decavanadate having no V_4. The rate of reduction to V^{IV}, measured by the increase in the absorbance at the peak, 700 nm (A_{700}), is 500 times faster with V_{10} at equivalent concentration of vanadium. This high rate was obtained in hepes and phosphate buffers (*p*H 7.0) (lines iii, iv).

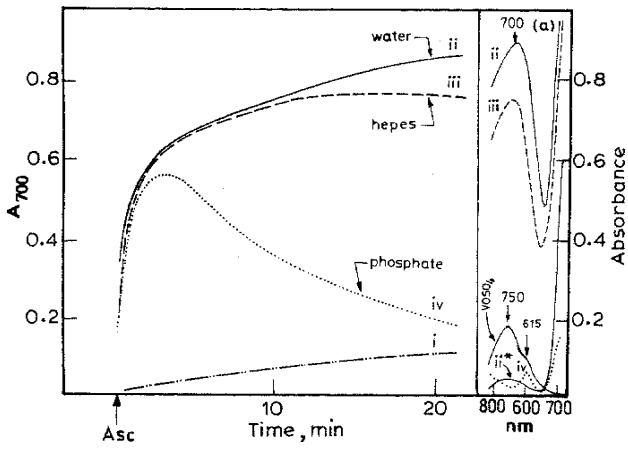

Figure 4. Reduction of vanadate and decavanadate (V_{10}) by ascorbate. The absorbance of reduced form, V^{IV} (A_{700}) was recorded after adding ascorbate (Asc) (1 mM) to an aqueous solution of metavanandate (10 mM), line i) or decavanadate (V_{10}) (1 mM line ii). Where mentioned 50 mM buffers (*p*H 7.0), HEPES (line iii) or phosphate (line iv), were used. The spectra of solutions ii-iv are shown in inset along with that of the standard $VOSO_4$ (10 mM). Note its low absorbance. On adding acid (20 mM HCl) absorbance of line ii) decreased (line ii*) (adapted from Aparna Rao & Ramasarma 2000).

After reaching a maximum, the absorbance (A_{700}) in hepes buffer and water stayed for more than 10 min indicating its stability. In phosphate buffer it declined possibly because of shift in its absorbance peak to 600 nm (see figure 3) and could easily be mistaken for reoxidation. The peak at 700 nm was without the shoulder at 615 nm, obtained with $VOSO_4$. On acidification, the A_{700} dropped nearly to 5%, the peak shifted to 750 nm, and the shoulder at 615 nm appeared. Maximum reduction was obtained with ascorbate : V_{10} ratio of 1:1 and at higher ratio, ascorbate produced no further change. As ascorbate is a 2-electron donor, this points to reduction of only two vanadium atoms in V_{10}. The blue compound, possibly V^{V}_{8}-V^{IV}_{2}, was stable at *p*H 7.0 for several hours and can be isolated in crystalline form on adding cold ethanol. Addition of H_2O_2 or diperoxovanadate could reoxidize it to the yellow-orange V_{10}. Thus, the two vanadium atoms in the V_{10} cage can undergo reversible redox changes and this structure-bound vanadyl form gains over 20-fold higher absorbance. A new dimension for vanadium reactivity is added.

Vanadyl is a radical and can be detected by its 8-banded spectrum in electron spin resonance (ESR) (figure 3, right panel). In phosphate buffer (*p*H 7.0) it was ESR silent (owing to oligomerization?), and the spectrum (and also possibly monomeric form) was restored in presence of a chelating agent, EDTA (Aparna Rao & Ramasarma 2000).

Enzymic Reduction of Vanadate

Erdmann et al. (1979) first reported disappear-ance of NADH upon addition of vanadate and the reaction was catalyzed by cardiac cell membranes. This activity was interpreted as NADH-vanadate reductase, wherein electrons of NADH were used to reduce V^{V}. Vyskocil et al. (1980) reported a non-enzymic metavanadate-dependent disappearance of NADH at a high rate with a *p*H optimum at 7.0. This reaction is a part of the overall non-enzymic reaction of vanadate-dependent oxidation of NADH by O_2 with extremely high rates in acid *p*H (Ramasarma et al. 1980), also catalyzed by an enzyme present in liver plasma membranes (Ramasarma et al. 1981). Using erythrocyte membranes as the enzyme source, the reaction was found to be specific for polyvanandate (later identified as V_{10}) and the reduction to V^{IV} was confirmed by appearance of 8-band ESR signals

(Vijaya et al. 1984). Further studies found an enzyme present in microsomal membranes also reduced polyvanadate (Patole et al. 1986). Delinked from oxygen consumption, the absorbance at 650 nm of this V^{IV} was stable for over 10 min (Patole et al. 1987). NADH-decavanadate reductase is therefore a functional part of the NADH-O_2 redox system (Meera Rau et al. 1987). In contrast to the marked inhibition of oxygen consumption, V_{10} reduction is insensitive to SOD and EDTA. The reduced form produced by the enzymes (as in the case of ascorbate) had several-fold higher absorbance compared to $VOSO_4$.

Some flavoproteins, such as bovine intestinal glutathione reductase, *Clostridial* lipoyl dehydrogenase and spinach ferredoxin-NADP oxidoreductase, showed activities that imply SOD-insensitive reduction of vanadate, and V^{IV} was identified by 8-band ESR spectrum confirming vanadate reductase activity (Shi & Dalal 1990, 1991). Isocitrate dehydrogenase obtained form pig heart is a well-known NADP-specific enzyme. This protein was found to have an alternative activity of using NADH to reduce V_{10}, but not metavanadate. Similar to ascorbate reduction, only a pair of vanadium atoms in V_{10} is reduced to a form having a peak at 700 nm with about 23-fold higher absorbance per reduced vanadium atom. The reduced form can be reoxidized to original V_{10} by H_2O_2. The activity was sensitive to heat but not SOD or EDTA, as expected of a vanadate reductase (Aparna Rao & Ramasarma 2000).

Vanadium enters through anion channel as V^{V} in tunicate vacuole against a 10^8-fold higher concentration gradient in seawater, accumulates as V^{III} on reduction by some unidentified strong reductant and thus restrained to leave the cell. None of the biogenic reductants including NADPH can do this even in acidic medium and tunichrome-polyphenol appeared to be effective (Ryan et al. 1996). Formation of V^{III} normally requires a strong reductant such as hydrogen and exclusion of oxygen, and a strong radical species such as a phenolate or NAD• would do the job. Vanadium being present in a complex [e.g., salen, N,N'-ethylenebis (salicyldeneamine) (Liu & Anson 2000)] seems to facilitate its reduction. We surmise that a peroxo-V-V dimer complex (not meta-V) and NADH (not NADPH) are the active species, and strong NAD• radical is likely to be the reductant of V^{IV} in our experiments. The V^{III} form is autoxidizable at neutral *p*H, unlike V^{IV}. A sample of V^{III} solution introduced into a medium containing phosphate buffer (50 mM, *p*H 7.0) in an oxygraph consumed oxygen in less than 10 sec. It readily complexes with O_2 yielding peroxovanadate [$V^{V}(O_2)$], as shown in the case of a salen derivative (Liu & Anson 2000), yet another way of consuming oxygen. This reaction is now adapted in our studies on polyvanadate-dependent oxidation of NADH by O_2, as it fits well with the product of O_2 reduction being a peroxide, but not H_2O_2 (Meera Rau et al. 1987). The reactions described above for the formation of decavanadate and reduction of vanadate are set into a pathway in figure 5.

Autoxidation of Vanadyl (V^{IV}); When Does it Occur?

An aqueous solution of vanadyl sulfate ($VOSO_4$) is acidic (*p*H about 3.0) and is stable over long periods at room temperature and in cold. At *p*H 8.0 and above vanadyl was oxidized in few hours, its blue color being replaced by yellow on conversion to vanadate (V^{V}). We found it was stable for several days at room temperature when its solution, adjusted to *p*H 7.0 by adding dilute alkali, was left in a petri dish exposed to air. Its spectrum in the visible range with absorbance peak at 750 nm and

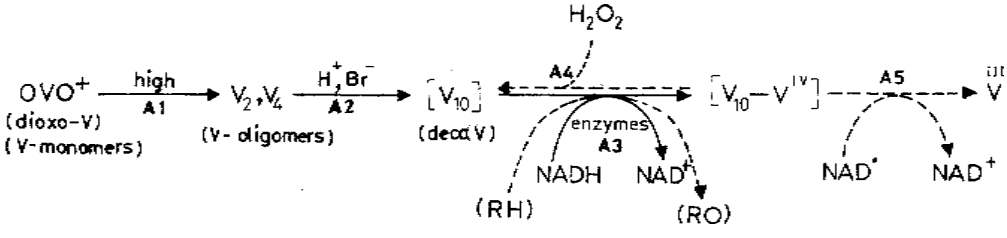

Figure 5. Oligomer of vanadate and its reduction. A1, oligomerization of monomer V at high concentration to V_2,V_4; A2, conversion of V_2,V_4 to decamer,V_{10} in acid medium, aided by Br^- ions; A3, reduction of V_{10} by enzymes using NADH, or chemically by reductants (RH); A4, reoxidation of V_{10}-V^{IV} by H_2O_2 to V_{10}; A5, reduction of V_{10}-V^{IV} to V_{10}-V^{III} by NAD• radical (assumed, based on experiments of Yamamoto et al. 1996 and Liu & Anson 2000)

the 8-band ESR spectrum were retained in such aged solutions. Phosphate buffered (*p*H 7.0) solutions of vanadyl sulfate showed no decrease in absorbance or consump-tion of oxygen during equilibration usually for 2-4 min before adding other reagents (Ravishankar & Ramasarma 1993, 1995, Ravishankar et al. 1994, 1996). This is our experience in many experiments with time extended for 30 min in some cases. Significant autoxidation could not have occurred during the few minutes when reactions under study were completed. It is important to use recrystallized vanadyl sulfate to remove any trace of impurity.

Misconception that a neutral aqueous solution of vanadyl (V^{IV}) is autoxidized is widespread, leading to free implication of $O_2^{\bullet -}$ in other reactions (for example, Liochev & Fridovich 1990). It seems to be based on an experiment on loss of absorbance at 766 nm of 75% in about 40 min of vanadyl sulfate solution (0.4 mM) in piperazine-N,N-bis(2-ethane sulfonic acid (PIPES)/Tris buffer (*p*H 6.1) (North & Post 1984). In view of cloudy precipitate formed in these reaction mixtures, possibly due to vanadyl-piperazine complex, aliquots were taken at intervals, acidified and absorbance measured. This was taken to represent autoxidation. The product, vanadate, or consumption of oxygen was not monitored in this experiment. It is necessary to check whether absorbance and peak of vanadyl complexes with such organic compounds changed as with phosphate buffer (see figure 3). It is essential to show oxygen consumption. And this we could not find in our experiments with vanadyl in phosphate buffer.

Oxidation of Vanadyl (V^{IV}) by H_2O_2

Vanadyl (OV^{2+}) is oxidized readily by H_2O_2 in acidic or neutral medium. Brooks and Sicilio (1971) described the underlying reactions in their classic paper. Very few of recent workers cited this paper published in 1971 (therefore not accessible on computer). Had investigators working on vanadyl oxidation not missed this paper, some contradictions that have crept into the literature could have been avoided. With a ratio of V^{IV}: H_2O_2 of 2:1, vanadate is the product, transiently forming hydroxyl radical ($^{\bullet}OH$). The radical formation was later confirmed by 1:2:2:1 ESR signals of adduct with spin trap DMPO, in acid medium by Carmichael (1990) and in phosphate buffer (*p*H 7.0) by Ravishankar et al. (1994). An addition complex ($OVOOH^{+}$) is first formed between OV^{2+} and HOOH, releasing a proton. The unstable VOOH-complex then breaks to yield V_1 (normally shown as doxovanadium, OVO^{+}, for convenience) and $^{\bullet}OH$. This is an important model reaction, applicable for other peroxo-bridged complexes (see below). The $^{\bullet}OH$ then attacks monoperoxovanadate [$OV(O_2)^{+}$] present in such acid reaction mixtures to yield a new oxy-radical intermediate (I) suggested to be $OVOO^{\bullet 2+}$. Appearance of compacted signal in kinetic ESR within the 8-band spectrum of vanadyl (figure 6, spectrum 2 in the middle) led to discovery of this new radical species (Brooks & Sicilio 1971).

Dioxygen (O_2) is a product of the overall reaction when vanadyl was in excess of H_2O_2. It means that a pair of oxygen atoms originating from H_2O_2 loses two electrons to other acceptors. An ingenious mechanism of dismutation of the radical, $OVOO^{\bullet 2+}$, with one peroxo-group reverting to oxygen, was suggested. Internal rearrangement of two electrons would reduce the other peroxide-oxygen atoms. They also showed that the oxygen release and the ESR spectrum of the new radical were absent in the presence of methanol and EDTA. Brooks and Sicilio (1971) explained all these with supporting data, and the reactions are set into a pathway in figure 7.

Addition of a variety of organic compounds blocked this oxygen release (e.g., ethanol, NADH, bromide, benzoate). These are oxidized instead by an intermediate in the pathway with consumption of O_2 (Ravishankar & Ramasarma 1993, 1995, Ravishankar et al. 1994, Aparna Rao et al. 1996). All these reactions with vanadyl and H_2O_2 were reproduced in our laboratory in phosphate buffer at

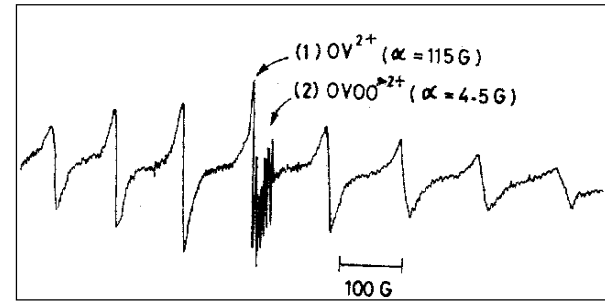

Figure 6. ESR spectra of vanadyl (OV^{2+}) (1) and intermediate [I], $OVOO^{\bullet 2+}$ (2). Both are 8-banded with differing a-values as shown (adapted from Brooks & Sicilio 1971).

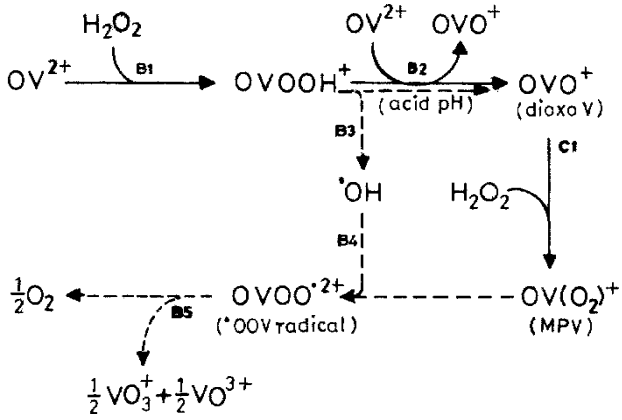

Figure 7. Oxidation of vanadyl (OV^{2+}) by H_2O_2 in acid medium (adapted from Brooks & Sicilio 1971). B1, addition product $OVOOH^+$; B2, oxidation of second OV^{2+} to produce two moles of OVO^+; B3, breakdown of addition complex to •OH radical in acid medium (broken lines); B4, generation of •OOV radical by oxidation of MPV; B5, dismutation of •OOV radical releases O_2.

*p*H 7.0, bringing out the significance of diperoxovanadate in substituting for H_2O_2.

Peroxo-complexes of Vanadium

Redox capability of vanadium is diversified by complexing with H_2O_2. Peroxo-complexes of the type VOOH (monodentate), $V(O_2)$ (bidentate) and VOOV (μ-peroxo) are formed from vanadate. The classic studies by Brooks and Sicilio (1971), Howarth and Hunt (1979), Harrison and Howarth (1985) and by Jaswal and Tracey (1991) provided model reactions that helped in our formulating peroxovanadium redox pathways. Two new oxo-peroxo radicals were discovered during these studies: $OVOO^{\bullet 2+}$ by oxidation of monoperoxo-V in acid medium (Brooks & Sicilio 1971), and $^{\bullet}OV(O_2)^{2+}$during breakdown of the unstable O-O bridge of μ-peroxo-V_2 at *p*H 7.0 (Ravishankar & Ramasarma 1995). These radicals are likely to interchange and be selective hydrogen-abstracting agents.

Vanadate, vanadyl and V_2O_5 undergo favorable condensation reaction with peroxo-group of H_2O_2 (HOOH),and also of diperoxovanadate $[HOOV(O_2)^{2+}]$. The products formed depend on *p*H, concentrations and ratio of H_2O_2 : V. These were characterized by ^{51}V-NMR spectra (table 2). Monoperoxovanadate (MPV) species appears in low H_2O_2 : V ratio and acid *p*H (< 3.0). Diperoxovanadate (DPV), the physiological peroxo species, is formed with H_2O_2 : V at a ratio of 2:1 in

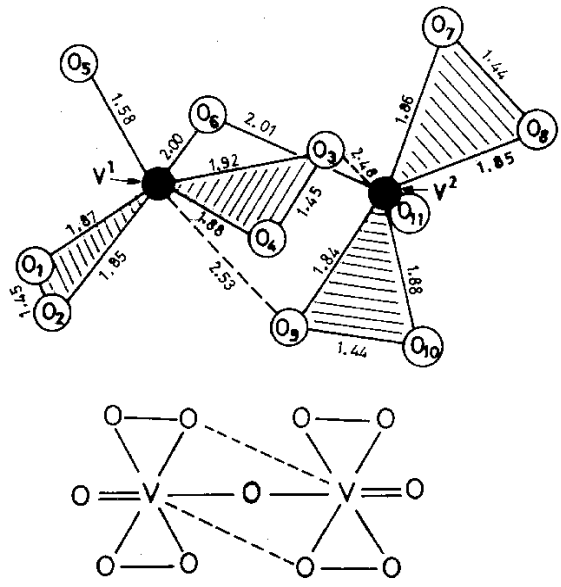

Figure 8. Crystal structure of dimeric ammonium vanadate. Note the shaded bidentate peroxo groups (adapted from Svensson & Stomberg 1971).

the broad *p*H range of 3.0 -8.0. From the formulae shown in the table 2, one of the two peroxo-groups seems to be -OO^-. Presence of hydroperoxo form, needed for complexing and redox reactions, is known in peroxovanadates (for more information see Casny et al. 2000). The first peroxo group is normally shown in the linear form, and the second, accompanied by release of protons, as bidentate, shown as "(O_2)". At high ratio and *p*H > 8.0 the triperoxovanadate (TPV) species dominate. Thus mono-, di- and tri-peroxo species are the preferred forms present in acid, neutral and alkaline *p*H, respectively. The crystal structure of ammonium diperoxovanadate $[(NH_4)_2V_2O_{11}]$ confirmed the presence of bidentate form in both peroxo-groups with O-O distance of 1.45Å compared to V-O distance of 1.87Å (figure 8). Interestingly, one of the bidentate oxygen atoms shares an unusual seventh coordination with the next V-atom in the solid state (Svensson & Stomberg 1971). Does this lead to linearization of one bidentate peroxo group when the dimer breaks in solution.

Addition of vanadyl to DPV at 1:1 ratio results in complete loss of DPV (-701 ppm) and the appearance of V_2 (-570 ppm) and cyclic-V_4 (-578 ppm), as determined by the changes in ^{51}V-NMR spectrum, and also by loss of 8-band spectrum of vanadyl in ESR. Measured in the oxygraph, molecular oxygen was released into the medium,

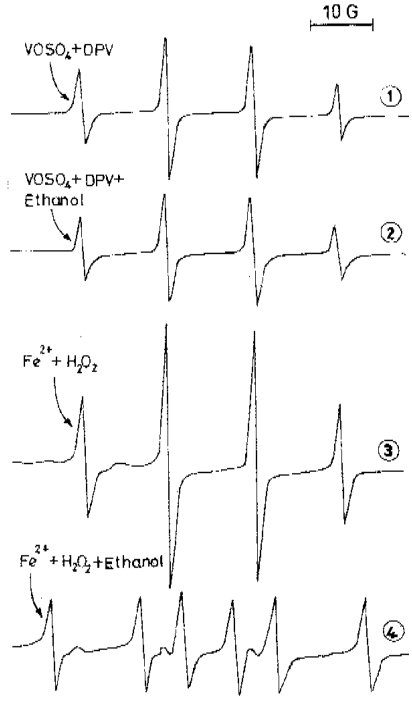

Figure 9. ESR spectra DMPO adducts of the radicals formed during oxidation of vanadyl by diperoxo-vanadate. The reaction mixtures contained DMPO (100 mM) in phosphate buffer (50 mM, *p*H 7.0) and, where mentioned, vanadyl (1 mM), DPV (4 mM) and ferrrous ammonium sulfate (1 mM), H_2O_2 (1 mM) and ethanol (170 mM). (adapted from Ravishankar & Ramasarma 1995)

equivalent to one-half of the amount DPV added (Ravishankar & Ramasarma 1993, 1995). The oxygen release is similar to addition of H_2O_2 but H_2O_2 was not formed as an intermediate with DPV as the oxidant. If that were the case, •OH radicals or the derived hydroxyethyl radicals in presence of ethanol, found with H_2O_2, should not be present. This was studied by the ESR spectra of a reaction mixture of vanadyl and DPV obtained in the presence of the spin trap, DMPO, without and with ethanol. It can be seen from figure 9 that the 1:2:2:1 quartet signal ($\alpha_N = \alpha_H$ = 14.9 G typical of DMPO-OH adduct) was obtained that remained stable in a high concentration of ethanol (170 mM) (line 1,2). Compare these with the signals obtained in the standard •OH radical-generating system of a mixture of ferrous ammonium sulfate and H_2O_2 where the signal was split into triplets of doublets by ethanol (line 3,4). This evidence led us suggest that the labile m-peroxo bridge of the addition complex of vanadyl and DPV [$OVOOV(O_2)^{3+}$] breaks to generate the oxygen radical, •$OV(O_2)^{2+}$. Oxygen release can be fitted with a dismutation reaction of this radical. These are based on reactions proposed by Brooks and Sicilio (1971) occurring in acid medium at the level of $OVOOH^+$ for formation of •OH and $OVOO^{\bullet 2+}$ radicals and for oxygen release. The final product of vanadium at neutral *p*H was some form of oligomer that would not recycle unlike V_1 (VO^{3+}) and mono-peroxo-V (VO_3^+), and so is represented as ½[VOV]. These are assembled into a pathway (figure 10).

Slow decomposition of H_2O_2, observed from an equimolar mixture of vanadate and H_2O_2 (which will have equal amounts of DPV and V_1), was interpreted as vanadium-catalyzed reaction (Jaswal & Tracey 1991). From the discussion, above it is clear that oxygen release will depend on vanadyl-complex. Slow formation of vanadyl from free, unbound V_1 is known to occur in acid medium. Also to be noted is that oxygen release corresponded to half of DPV present and not unlimited decomposition of H_2O_2.

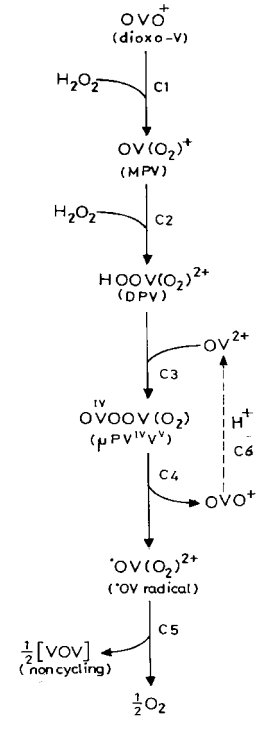

Figure 10. The backbone reaction sequence of vanadate-H_2O_2 interaction and release of O_2 on reduction by vanadyl. C1,addition of first H_2O_2 to make monoperoxo-vanadate (MPV); C2, addition of second H_2O_2 to form diperoxovanadate (DPV), stable at *p*H 7.0; C3, addition complex of vanadyl and DPV, transiently retaining m-peroxo bridged V-V; C4, internal oxidation and release of OVO^+ and •$OV(O_2)^{2+}$ radical; C5, dismutation of •OV radical to release O_2 and [VOV], a form of non-cycling vanadate. (adapted from Arpana Rao et al. 1990).

Vanadate-dependent Oxidation of NADH

Oxidation of NADH stimulated by polyvanadate (V_{10} + excess vanadate) (Ramasarma et al. 1980, 1981, Vyscocil et al. 1980) was first reported in the early 1980's. Later, vanadyl in the presence of H_2O_2 (Liochev & Fridovich 1991) and DPV in place of H_2O_2 (Ravishankar & Ramasarma 1995) were also found to be effective. Enzymes present in several membranes considerably enhanced the rate of this reaction with vanadate. These include: cat ventricles (Erdmann et al. 1979), pig erythrocytes (Crane et al. 1980), mouse liver plasma membranes (Ramasarma et al. 1981) rat liver microsomes (Menon et al. 1980), rat erythrocyte membranes (Vijaya et al. 1984), sugarbeet microsomes (Briskin et al. 1985), bovine cream xanthine oxidase (Darr & Fridovich 1984), and yeast plasma membranes (Minasi & Wilsky 1991).

The following characteristics of this unusual NADH oxidation reaction were defined from the early work on plasma membranes and liver microsomes from our laboratories. A phosphate buffered solution of NADH showed small non-enzymic oxidation on adding polyvanadate (Ramasarma et al. 1980) or vanadate (Vyskocil et al. 1980) which was enhanced by enzymes in membrane preparations. NADPH had similar non-enzymic activity as NADH (Vijaya & Ramasarma 1984), but only 1/10 to 1/30 of enzymic acitivity in the membranes tested. Activity increased as the *p*H decreased. Phosphate or any other anion is required (Ramasarma et al. 1981). NADH : O_2 ratio was 1:1 indicating a 2-electron reduction of O_2 to H_2O_2, confirmed by the release of half of consumed O_2 on addition of excess catalase as expected of a peroxide product (Vijaya et al. 1984, Meera Rau et al. 1987) (figure 11). The slow release of O_2 and the need for a large amount of catalase is typical of DPV (Ravishankar et al. 1995), whereas H_2O_2 is 100-fold more active. Presence of DPV in such reaction mixtures was confirmed by the characteristic chemical shift at -695 ppm in ^{51}V-NMR spectra.

It should also be remembered that there could be differences when the activity is measured by the disappearance of NADH (decrease in A_{340}) or by consumption of O_2. The activity of NADH oxidation was inhibited by EDTA, cyanide, mannitol, histidine, ascorbate, noradrenaline, adriamycin, cytochrome c, Mn^{2+}, horseradish peroxidase, catalase and SOD (Meera Rau et al. 1987). The sensitivity to these inhibitors was markedly higher when measured by O_2 consum-ption as compared to NADH disappearance because NADH is used for reduction of V_{10} and O_2. V_{10} reduction is an essential part-reaction, insensitive to these inhibitors that could be delinked from the overall O_2 consuming reaction. A solution of poly-vanadate obtained by extraction of solid V_2O_5 with alkali containing a mixture of vanadate oligomers (V_2, V_4, V_{10}), but not metavanadate (V_1, V_2, V_4), was most active (Vijaya et al. 1984, Meera Rau et al. 1987). Short-lived controversies on the phosphate requirement and metavanadate being active form (see Darr & Fridovich 1984) arose; later studies in our laboratory resolved these by showing that phosphate gives high rates (Patole et al. 1988), and that both V_{10} and metavanadate (V_1) were required (Kalyani & Ramasarma 1992). Maximum activity, several times higher than the sum of two activities, was obtained when the V_1 : V_{10} ratio was in the range of 1-5 (figure 12). The dependence on phosphate, V_1 and V_{10} for maximal activity is striking, absence any one reduces the rate enormously.

A novel phenomenon was observed in experiments on oxidation of NADH by microsomes with pure V_{10} (free of V_1 as judged by NMR data) (Kalyani & Ramasarma 1993). After an initial phase of small rate, the reaction automatically shifted into a "burst phase" of O_2 consumption that rapidly oxidized all the available NADH (figure 13). This provides ideal model reactions for "respiratory

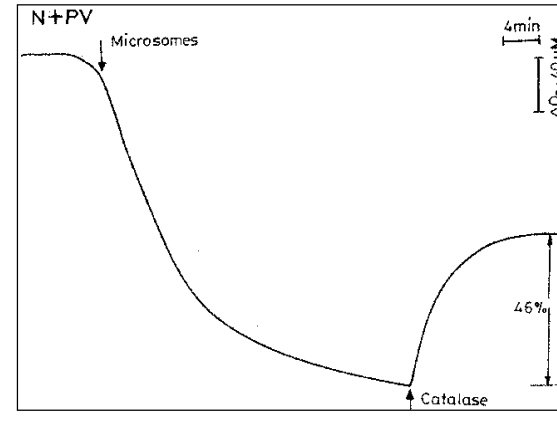

Figure 11. Changes in the dissolved oxygen during polyvanadate- stimulated oxidation of NADH by microsomes and its reappearance on adding catalase. The reaction mixture contained phosphate buffer (50 mM, *p*H 7.0), NADH (0.2 mM) (N), polyvanadate (0.1 mM) (PV); rat liver microsomes (20 μg protein/ml) and catalase (100 μg protein/ml), as indicated by arrows. (adapted from Meera Rau et al. 1987).

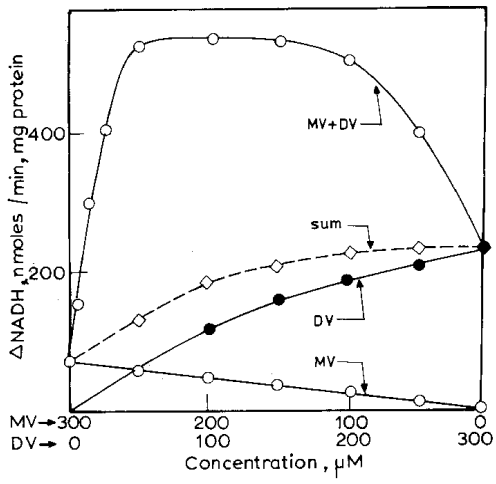

Figure 12. Effect of varying the ratio of metavanadate (MV) and decavanadate (DV) on stimulation of the rate of oxidation of NADH (0.1 mM) by rat liver plasma membranes (24 μg protein/ml). Note the rates obtained by the mixture are several-fold higher than the sum of the two when the ratio is between 1-5. (adapted from Kalyani & Ramasarma 1995).

burst" in phagocytosis wherein the product, H_2O_2, kills the invading organisms. The finding that pervanadate (a mixture of DPV, vanadate and V_{10}) added to neutrophils activates NADPH oxidase independently of protein kinase C (Yaname et al. 1999) supports this. Note in figure 13 the unambiguous requirement of phosphate anion for starting the burst and its sensitivity to SOD. The pre-burst phase was apparently used for reducing V_{10} and the burst point, reached after reduction of two V-atoms of V_{10} as discussed earlier, must have provided the essential free V_1. Confirming this, metavanadate added in the middle of this phase yielded a high rate. Implicit in these effects is the possibility that free vanadate, or more precisely phosphovanadate, parks the peroxide product of O_2 reduction as DPV. It appeared that V_{10} participation depends its capability to form addition complex with DPV, and regenerating itself by shedding the peroxo-group it preserved. The proposed reactions are set into figure 14.

Would vanadyl (V^{IV}) replace V_{10} in stimulating NADH oxidation? Liochev and Fridovich (1987b) reported this with $V^{IV} + H_2O_2$. This was confirmed in our laboratory and phosphate buffer is also required for this reaction (Ravishankar et al. 1994b). These reagents form addition complex followed by break of its m-peroxo bridge. The •OV-type radical thus formed dismutates releasing O_2 (see figure 10).

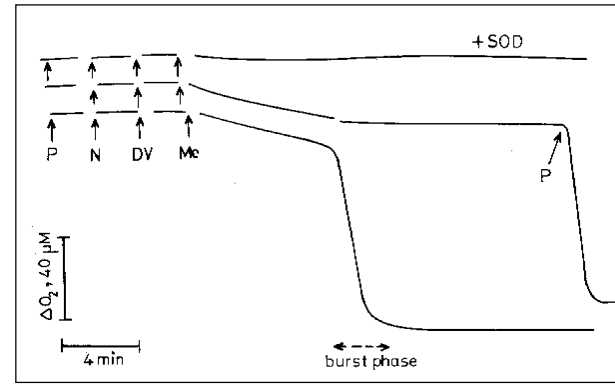

Figure 13. Burst of oxygen consumption during oxidation NADH in the presence of pure decavanadate. The reaction mixture contained phosphate buffer (50 mM, *p*H 7.0)(P); NADH (0.2 mM) (N), decavandate (0.4 mM as V) (DV); rat liver microsomes (25 μg protein/ml) (Mc); and where mentioned superoxide dismutase 10 μg protein/ml) (SOD). Note the burst reaction does not occur until phosphate is added (middle line). (adapted from Kalyani & Ramasarma 1993)

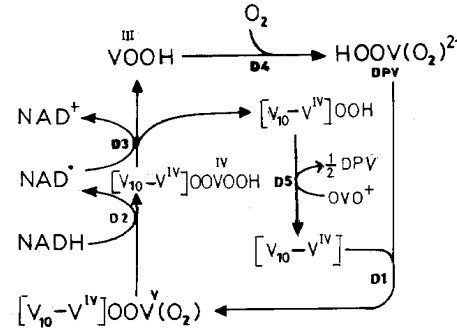

Figure 14. Proposed peroxovanadate cycle for NADH oxidation stimulated by decavanadate (V_{10}). D1, addition complex of [V_{10}-V^{IV}] and DPV; D2, reduction of V^V in the complex to V^{IV}; D3, further reduction of V^{IV} to V^{III} by NAD• radical, a strong reducing agent' D4, addition of O_2 to V^{III} forming the oxidation product $V^V(O_2)$; D5, recovery of peroxo group from the intermediate and regenerating [V_{10}-V^{IV}] sufficient in catalytic amounts. Note the essentiality of vanadate is in accepting peroxo group of reduction product of O_2, without which the cycle can not continue.

NADH added after O_2 release remains untouched. But present during the reaction between the reagents, NADH stops O_2 release, uses one of the intermediates for its own oxidation that ultimately gives a ratio of NADH : O_2 of 1:1. Addition of catalase released O_2 into the medium to the extent of 20% with H_2O_2 and only 2% with DPV (figure 15) in contrast to the 50% release in the polyvanadate experiments. The reduced form of O_2 in the latter case was certainly not a peroxide. The stoichiometry of NADH : V^{IV} : O_2 was 1:2:1. This indicated that the 4-electrons available from NADH and 2- V^{IV} might fully reduce oxygen atoms of O_2 which in turn end

up as part of the product OVO^+. DPV must be recycling because it was essential and effective at a very small concentration. The proposed reaction cycle is shown in figure 16.

Are $O_2^{\bullet-}$ and $^\bullet OH$ Radicals Involved in Vanadate-dependent NADH Oxidation?

It is easy to accept the entrenched dogmas that 2-electron reduction of O_2 goes through the step of superoxide followed by its dismutation, and the powerful hydroxyl radical can oxidize NADH. But the evidence available is not convincing.

We looked for $O_2^{\bullet-}$ by ESR spectrum of its DMPO adduct in the reaction mixture of polyvanadate-dependent NADH oxidation. We found the 1:2:2:1 quartet signal typical of $^\bullet OH$ radical, at that time considered evidence for initial formation of DMPO-OOH (Vijaya & Ramasarma 1984, Vijaya et al. 1984). Later experiments showed that this was due to another radical, possibly $^\bullet OV(O_2)^{2+}$ (Ravishankar & Ramasarma 1995). To my knowledge there is no other direct evidence for $O_2^{\bullet-}$ formation in these reactions.

The crucial finding used by many was our observation of marked inhibition by SOD of vanadate-dependent oxidation of NADH by O_2 (Ramasarma et al. 1981). How to explain anomaly of the inhibition of generation of H_2O_2 (thought at that time as the product) by SOD, the enzyme that forms H_2O_2 by dismutation of superoxide? SOD should have helped speed up the process instead. In our early attempts we did consider $O_2^{\bullet-}$ as the intermediate (Vijaya et al. 1984, Patole et al. 1988), accepting that SOD inhibition must be interpreted as superoxide involvement in the reaction. In this scheme, O_2 receives the first electron from $NAD^\bullet$ radical (a known reaction) and the second from V^{IV} (Vijaya et al. 1984) (figure 17, left panel). But this was unsatisfactory with respect to implication of V_{10} and SOD inhibition. Superoxide-based explanation of SOD inhibition was offered according to which a "hypothetical and clearly very unstable" primary oxidant of NADH, V^{IV}-OO, is formed from V^V and $O_2^{\bullet-}$" (Darr & Fridovich 1984) (figure 17, middle panel). An incomprehensible proposal since the products can be either $V^{IV}+O_2$ (electron transfer) or V^V-$OO^\bullet$ (addition product). The concept of superoxide-initiated chain reaction and its termination with SOD was then introduced (Liochev & Fridovich 1986). Its length was calculated as the ratio of NADH disappeared to that of $O_2^{\bullet-}$ formed by xanthine oxidase reaction. Formation of $O_2^{\bullet-}$ and loss of NADH coincidentally occur but are they linked? We never appreciated the need for a chain reaction as a part of overall stoichiometric utilization of NADH and O_2.

One important feature of this enzyme protein was missed out. Oxidation of NADH by xanthine oxidase protein required only vanadate, but not

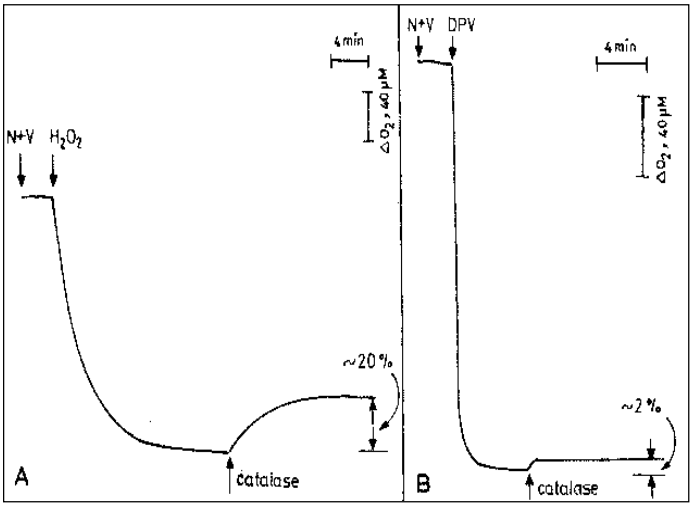

Figure 15. Oxygen consumption during oxidation of vanadyl. The reaction mixture contained phosphate buffer (50 mM, *p*H 7.0) and NADH (0.2 mM) (N). Other additions where mentioned: left panel, H_2O_2 (0.005 mM) and vandyl sulfate (0.1 mM) (V); right panel, DPV (0.01 mM) and. vanadyl sulfate (0.2 mM); catalase (60 µg protein/ml) (adapted from Ravishankar et al. 1994b, Ravishankar & Ramasarma 1995).

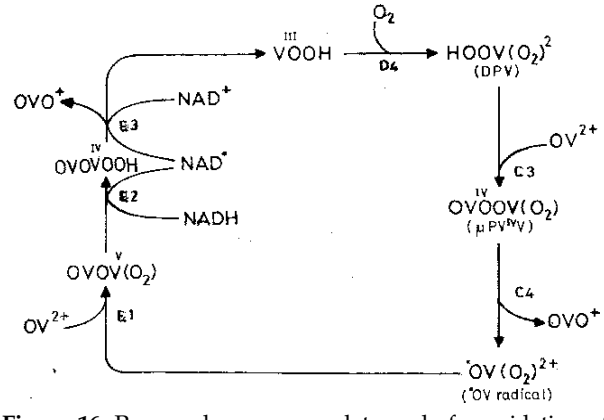

Figure 16. Proposed peroxovanadate cycle for oxidation of NADH and vanadyl (V^{IV}). C3 and C4 are from the backbone pathway (Figure 11) that will produce $^\bullet OV$ radical; E1, addition complex of this radical and another molecule of vanadyl to fit with the observed stoichio-metry; E2, E3 sequential reduction of $V^V \rightarrow V^{IV} \rightarrow V^{III}$ by NADH similar to D2, D3; D4 is the common step of reduction of O_2 by V^{III} to regenerate DPV and continue the cycle.

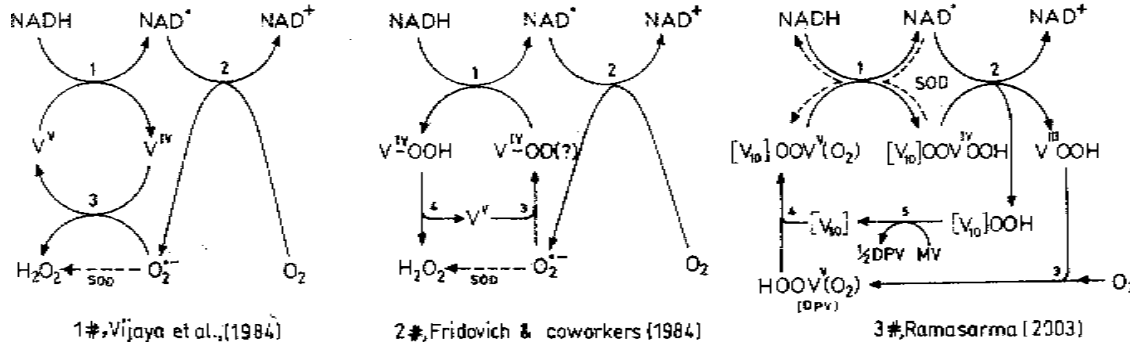

Figure 17. Proposed scheme for NADH oxidation by vanadate. Reactions 1 and 2 denote the removal of the two electrons from NADH. Vijaya et al. (1984) proposed that V^{V} is oxidant in 1# and O_2 in 2#. Darr and Fridovich (1984) proposed a hypothetical V^{IV}-OO (?) as the oxidant in 1#. Our latest proposal discussed in this review, shown on the right, has the major departure that both electrons from NADH go to reduce sequentially $V^{V} \rightarrow V^{IV} \rightarrow V^{III}$ and reduction of O_2 by V^{III} regenerates DPV to continue the cycle. It fits with V_{10} as the active species that undergoes redox and also vanadate requirement with acting as a sink of product, peroxide. SOD inhibition is marked at the very first step that would stun the reaction by reversing.

xanthine (Khandke et al. 1986). Therefore it is obvious that the two reactions of oxidation of NADH and xanthine are independent, intrinsic activities. Thus the large rate of superoxide generated by oxidation of xanthine was unnecessary, and in its absence vanadate-dependent oxidation of NADH occurs. Then whither $O_2^{\bullet -}$ in the mechanism?

Further complexities were introduced by bringing in •OH radicals, and "secondarily derived radicals" (hydroxyethyl?) (Liochev & Fridovich 1991). Hydroxyl radicals were no doubt formed under these conditions and are capable of destroying a variety of compounds including NAD^+ and DNA. Indication of their non-participation in NADH oxidation was indicated by lack of effect of a variety of •OH-scavenger compounds (Ramasarma et al. 1981, Stanikiewicz et al. 1991, Ravishankar et al. 1994). Do these radicals generated in another system without vanadate promote NADH oxidation? A simple experiment showed that •OH-radical can not oxidize NADH. The absorbance at 340 nm remained unaltered when NADH (0.2 mM) was present during generation of •OH radicals in the standard Fenton system of oxidation of ferrous ammonium sulfate (0.2 mM) by H_2O_2 (1 mM) (Ravishankar & Ramasarma 1995, Ramasarma & Ravishankar 2003). Based on the above findings, I have reservations in accepting involvement of $O_2^{\bullet -}$ and •OH radicals, and also the chain reaction, in the polyvanadate -dependent oxidation of NADH eventhough it is against the current. NADH oxidation is a characteristic of vanadium. Therefore we proposed alternative way of retaining reduced oxygen as its peroxide. The information in figure 14 is rearranged in figure 17 (right panel) to account for the electron transfer from NADH directly to V^{V} in oxo, peroxo complexes, and from V^{III} formed to O_2.

Peroxovanadate Cycles for NADH Oxidation Dependent on V_{10}- and V^{IV}

The acquired information is set in table 3 for reactions dependent on V_{10} (type I) and vanadyl (type II) accompanied by some assumptions. Reaction pathways are built for these (figure 14, 16) to fit with available evidence and assumptions (based on related reactions). Reactions E1 and C3 are based on the property of condensation of V^{IV} forms with peroxo-V and the products were inferred and not yet experimentally detected. Reaction E1 is a similar condensation to bring in the second V^{IV} to satisfy the stoichiometry. Reactions D2, E2 and D3, E3 are stepwise reduction of $V^{V} \rightarrow V^{IV} \rightarrow V^{III}$ embedded in divana-date complexes. This is a departure from all the previous proposals in that both electrons from NADH are given to a V-atom. V-atoms in divanadium salen derivatives can exist in mixed valency states of V^{V}-V^{IV} and V^{IV}-V^{III} and undergo reversible redox (Yamamoto et al. 1996). One additional assumption in reactions D3, E3 is that once reduced, V^{III} breaks away from the divanadium complex. Direct oxidation of V^{III}-OOH by O_2 forms bidentate addition to V^{III} to produce $HOOV^{V}(O_2)$ (reaction D4) and this proposal has support from the experiment of Liu and Anson (2000) wherein salen-V^{III} is converted to salen-$V^{V}(O_2)$ on oxidation by O_2. DPV is thus

Table 3. *Comparison of properties of NADH oxidation dependent on V_{10} and V^{IV}*

Property / Parameter	V_{10}- dependent (type I)	V^{IV}- dependent (type II)
Catalytic vanadium form	Decavanadate (V_{10})	Diperoxovanadate (DPV)
Consumed vanadium form	V^{V} (vanadate, OVO^{+}) needed to recover -OOH	V^{IV}(vanadyl) oxidized to vanadate
Fate of μ-peroxo complex and use of peroxo product	O-O group preserved as DPV	broken and used for oxidation of V^{IV}
Phosphate	required	required
NADH : O_2 ratio	1:1	1:1
Overall stoichiometry	NADH : O_2 : V_1 = NAD^{+} : ½ DPV	NADH : O_2 : $2V^{IV}$ = NAD^{+} : 2 OVO^{+}
Assumptions		
Oxidant of NADH	$[V_{10}\text{-}V^{IV}]OOV(O_2)^{2+}$	$V^{IV}OV(O_2)^{2+}$
Steps of electron transfer	$V^{V} \rightarrow V^{IV} \rightarrow V^{III}$	$V^{V} \rightarrow V^{IV} \rightarrow V^{III}$
Step of SOD inhibition	first electron transfer	first electron transfer
Break of V-V complex	once V^{III} formed	once V^{III} formed
Oxygen consumption	V^{III} to form $V^{V}(O_2)$	V^{III} to form $V^{V}(O_2)$

regenerated to continue the cycle. In type I, transferring a peroxo group to vanadate in reaction D5 regenerates reduced V_{10} [V_{10} -V^{IV}] and this will continue the cycle. The need for phosphate in the overall pathway is likely to be in all the steps with at least one phosphate group added to vanadium atom in place of a positive charge shown. Admittedly acceptance of this peroxovanadate cycle must await further evidence regarding mixed valency intermediates. A previous attempt has been made to implicate V^{III} (Stanikiewicz et al. 1991). Its complex with superoxide, V^{III} - $O_2^{\bullet -}$ acts as the oxidant of NADH, and the O-atom receives the electron according to this.

What about SOD inhibition that sparked all these studies? The larger rate of consumption of O_2 during vanadate-catalyzed NADH oxidation was always sensitive to SOD. Addition of SOD saves both NADH and O_2. This points to stopping the very first step, indicating D2,E2 as the possible sites of action of SOD. Notice the products of these reactions are $NAD^{\bullet}$ and V^{IV}, both radical species. Known for radical dismu-tation, SOD may reverse reactions D2,E2.

Vanadate Catalyzed Bromoperoxidation

Bromoperoxidases are involved in the biosyn-thesis of a variety of natural brominated products. This is the first enzyme found by ESR (de Boer et al. 1986) and NMR (Vilter & Rehder 1987) studies to contain protein-bound vanadium essential for the activity. The choice of vanadium in these proteins and its known ability to form complexes with H_2O_2 led to the implication of peroxovanadate as a bromide oxidant. By itself, H_2O_2 is capable of this 2-electron oxidation in acid medium (Clauge & Butler 1995) but is ineffective at physiological *p*H. The enzyme explicitly functions in catalyzing rate-determining bromide oxidation to HOBr which non-specifically transfers its Br atom to any acceptor (de Boer & Wever 1988).

The credit for first biomimetic model goes to Sakurai and Tsuchiya (1990). They found bromination of an acceptor occurred in phosphate medium (*p*H 6.0) containing excess H_2O_2 and KBr in presence of vanadyl sulfate, but not vanadate. Without the acceptor, oxygen was released. Notice similarity with story of NADH oxidation. They however assigned minor role to vanadium of merely holding Br-atom for oxidation by $^{\bullet}OH$ radicals, and strangely never followed it up. Using a mixture of metavanadate and H_2O_2 in acid medium, de la Rosa et al. (1992) showed progress-ive loss of pre-formed peroxovanadates during the course of bromina-tio.They strongly supported peroxovan-adate as the bromide-oxidizing species. About the same time, Bhattacharjee (1992) found a mixture of V_2O_5 and H_2O_2 was effective in aqueous medium, but not NH_4VO_3+H_2O_2,$NH_4[VO(O_2)_2H_2O]$and $NH_4[VO(O_2)_2$ (GlyH)]H_2O (Bhattacharjee et al. 1995). Initially, both mono- and di-peroxovanadate (MPV + DPV) were proposed as the bromide-

oxidants (Soedjak et al. 1995, Claugue & Butler 1995) since they were found in such mixtures and their NMR peaks decreased during bromination. Tiny amounts of a V-compound with broad resonanace at -670 ppm, assigned to a triperoxovanadiumcomplex,H[$(O_2)_2OVOVO_2(O_2)$] (Harrison & Howarth 1985), suggesting that this complex may be the "critical oxidant of bromide" formed by combining MPV and DPV. Peroxo-groups in this complex are no different from MPV or DPV; then the query how it gains oxidant activity remains unexplained. This minor constituent was indeed formed at high vanadate concentration in acid medium but we could not reproduce its loss in the presence of bromide (Sarmah et al. 2002).

A new characteristic emerged from our studies of bromide oxidation in phosphate buffer. Bromoperoxidation was negligible when a pure crystalline preparation of DPV was used, or on converting free vanadate completely into DPV in excess H_2O_2, and the activity was restored on adding vanadyl, or uncomplexed vanadate that converts to vanadyl. It looked reasonable to assume that an oxidant must have emerged out of interaction between vanadyl and DPV leading to inference that this may be the μ-peroxo-bridged complex, $OVOOV(O_2)^{2+}$. A cycle of reactions involving, mono-, di-, μ- and bromo-peroxovanadates was drawn up for forming the bromide oxidant and it recycling. For the first time a complete set of reactions with peroxovanadates is formulated operating in a cycle needing only H_2O_2 for each bromine atom transferred. Indeed, continuous bromination of phenol red was demonstrated experimentally with the glucose-glucose oxidase system supplying H_2O_2 (Aparna Rao et al. 1996) in a system containing metavanadate and KBr in phosphate medium (*p*H 5.5). The merit of this proposal is that it assigns the bromide-oxidizing ability to μ-peroxo group of H_2O_2 now retained in the vanadate dimer.

Chaudhuri and coworkers (Bhattacharjee et al. 1990, 1995) synthesized a diglycine derivative of triperoxodivanadate $[V_2O_2(O_2)_3(GlyH)(H_2O)_2]$ that had the distinguishing μ-peroxo-bridge between two V-atoms. This compound was found to be highly active in bromine transfer and liberating bromine gas in the absence of an acceptor (Bhattacharjee et al. 1995). Since this compound was closely related to the proposed intermediate, we further examined its reactivity. On adding the solid to the aqueous reaction mixture a stable A_{267}-compound (BrOH) was instantly produced which converted phenol red to bromophenol blue (A_{592}). However, its aqueous solution was ineffective, notwithstanding the fact that it retained 55% of vanadium in the peroxy form. Obviously, the activity of the original compound was limited to a short period after hitting the water and lasted until parting of the peroxo bridge into its constituents, MPV and DPV. Similar results were obtained with derivatives of triglycine (Sarmah & Islam 2001) and of peptides gly-gly, gly-ala and gly-asn as hetero-ligands (Sarmah et al. 2002). We ascribe this to ready loss of hydrogen bonding between ligands in water.

Any biomimetic of bromoperoxidation cannot ignore VOOV-type intermediate. Active research groups in the field (Clauge & Butler 1995, Hamstra et al. 1998), continue to promote bidentate form of peroxovanadates as the active oxidant. Notwith-standing awareness of the ineffectiveness of DPV, several authors have claimed "hydroperoxo complex" of vanadium as the active species (see Casny et al. 2000). Another overlooked defect is the absence of an acceptable catalytic cycle with regeneration of the crucial intermediates. Our

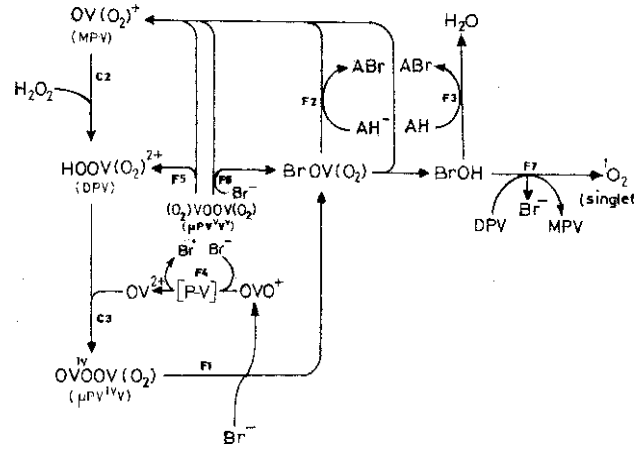

Figure 18. Proposed peroxovanadate cycle for bromoperoxidation. Cycle of reactions consists of peroxovanadates of mono- (MPV), di- (DPV), μ-peroxo-(μ-$PV^{IV}V$) and bromo- (BrPV) (the last two intermediates inferred). C2, C3 belong to the backbone pathway producing the proposed bromide oxidant, m-peroxo divanadate. F1, oxidation of bromide; F2, transfer of Br to acceptor AH; F3, BrOH, formed in the absence of AH, can transfer Br on subsequent addition of AH; F4, vanadyl regenerated from vanadate released in F1 by bromide; F5, synthetic m-peroxo-divanadate breaks down rapidly to MPV+DPV; F6, reaction F5 in presence of bromide yields bromo-PV capable of transferring Br to AH; F7, BrOH reduced to Br^- by DPV producing singlet oxygen.

proposed peroxovanadate cycle (figure 18) has all these features.

Crystal structures of haloperoxidase proteins are now available for *Curvularis inequalis* (Masserschmidt et al. 1997), *Ascophyllum nodosum* (Weynad et al. 1999) and *Corallina officinalis* (Isupov et al. 2000). These are dimeric proteins with superimposable subunits, each having a vanadium atom. The V-atoms in a protein are too far apart, at least 40 Å (Messerschmidt et al. 1997), to make a peroxo-bridged dimer. The enzyme protein from *A. nidosum* picked up an extra vanadium atom at a non-specific second site when treated with excess vanadate (Rehder et al. 1991). The enzyme protein treated with ^{17}O-enriched H_2O_2 showed a broad ^{17}O-NMR signal at 593 ppm, assigned to peroxo ligand in symmetric side-on coordinaton mode, and also a narrow signal at 412 ppm, assigned to free, unspecific diperoxo-vanadate, were found (Casny et al. 2000). But the authors dismissed this second vanadium as an "artefact due to partial release of vanadate from active centre by hydrogen peroxide". As these changes are dependent on H_2O_2 and bromide, they can represent the second vanadium atom undoubtedly necessary for activity in our experiments and proposal.

Oxidation of Ethanol and Benzoate by Peroxovanadate Compounds

Liochev and Fridovich (1991) reported an oxygen release reaction from a mixture of vanadyl sulfate (0.2 mM) and H_2O_2 (0.4 mM) in 20 mM phosphate buffer (*p*H 7.4), and also a "dioxygen consumption reaction followed by dioxygen evolution" when ethanol was present. But these effects of oxygen release in phosphate-buffered medium claimed by these authors are not reproducible, and they occur only when the buffer was omitted (Ravishankar et al. 1996). These reactions are in accord with the report of Brooks and Sicilio (1971) in acid medium and are characteristic of interaction of $V^{IV} + H_2O_2$ (or DPV). Only preformed DPV releases O_2 on addition of V^{IV} in phosphate buffer (Ravishankar & Ramasarma 1993; Ravishankar et al. 1994). Adding increasing amounts of ethanol progressively suppressed this reaction and increasing amounts of O_2 were consumed concomitantly. This is a general property of a wide variety of organic and inorganic compounds such as NADH, bromide, ethanol, methanol, propanol, octanol, mannitol, benzoate, formate, histidine, imidazole, citrate, and Tris (Ravishankar & Ramasarma 1993, Ravishankar et al. 1994, Ravishankar et al. 1996). Illustrated in figure 19 are concentration-dependent changes of O_2 from release to consumption for each compound.

A hydroxylation appeared obvious in view of concomitant oxygen consumption in such react-ions. The reaction mixture in the case of benzoate showed the presence of p-hydroxybenzoate and the amount of this product accounted for 80% of O_2 consumed. These results prompted us to propose a reaction pathway shown in figure 20 that consisted of hydrogen abstraction by the V-radical described

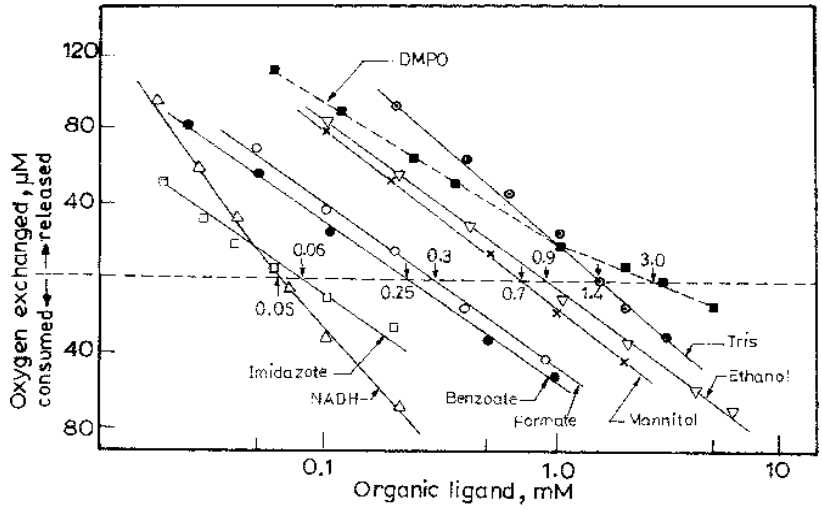

Figure 19. Oxygen exchange reactions in presence of organic ligands. The reaction mixture contained phosphate buffer (50 mM, *p*H 7.0), DPV (0.4 mM), $VOSO_4$ (0.4 mM) and increasing amounts of organic compounds. The change in the dissolved oxygen concentration was measured in an oxygraph.

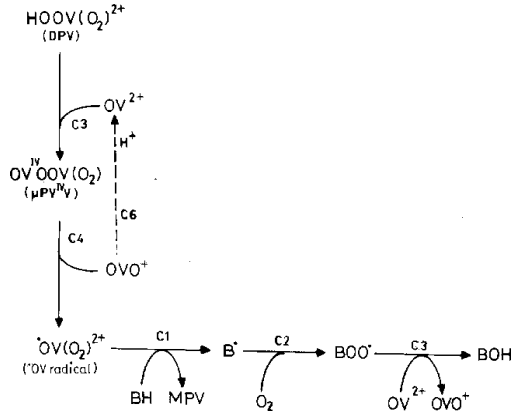

Figure 20. Oxidation of benzoate by diperoxovanadate and vandyl. C3, C4, C6 reactions are from the backbone pathway (Figure 11) for producing the •OV radical; G1, H-atom abstraction from benzoate by the radical; G2, attack of the B-carbon radical by O_2 to give BOO• radical; G3, reduction of this radical to BOH by $VOSO_4$ (3 moles?). (adapted from Ravishankar et al. 1994)

above, attack by O_2 to form benzoate-OO•, followed by reduction to the product by V^{IV}.

The oxidation product for ethanol was found to be acetaldehyde measured by the specific enzyme, alcohol dehydrogenase. The ratio of acetaldehyde: O_2 reached near unity at high ethanol concentration (10 mM). When phosphate buffer was omitted the medium was acidic and under these conditions no acetaldehyde was found.

The Importance of being Peroxovanadium

A number of cellular phenomena have been identified with known participation of H_2O_2, such as respiratory burst, egg fertilization, parasite maturation, hormonal response, membrane transport, cyanide-resistant respiration, lignification and alternative oxidase and thermogenesis. Many direct effects of H_2O_2 on cells, tissues and proteins have been reported (see Ramasarma 1990 for a review) and the number is increasing. To observe these effects H_2O_2 had to be added at mM concentration, understandable in view of its loss by scavenging by highly active catalase and glutathione peroxidase. Then how can H_2O_2 act in peroxidations? It will be no surprise if many of them involve DPV to gain efficiency at low cellular concentrations and the actions explained by oxidation of proteins of some of the intracellular enzymes.

To satisfy cellular need of peroxidation reactions, natural peroxide formed from H_2O_2, stable to degrading enzymes and capable of offering peroxo group must be present. DPV fits remarkably in such a role. Vanadate sequesters H_2O_2 in competition with catalase at neutral *p*H and the product DPV is at least 50-times more stable to degradation by this powerful enzyme. Its peroxo groups are functional in 'user' peroxidase reaction and in a regulatory inactiva-tion of an enzyme. Invariably DPV is at least 100-times more effective (Aparna Rao et al. 1998). Testing DPV as a possible, efficient peroxide substrate is therefore recommended.

A mixture of vanadate and excess H_2O_2 ('pervanadate') was found to have interesting effects on several enzymes. This pervanadate is found to have predominantly DPV and varied amounts of vanadate depending on the time allowed for catalase treatment. Most workers used this step of treatment with large amounts of catalase intended to remove excess H_2O_2, which would have produced back some free vanadate by catalase action on DPV (Ravishankar et al. 1995), and one should be aware of additional actions of free vanadate. The reactions affected by DPV are listed in table 4. Interestingly, some of these are signal-transducing systems. We anticipate many more effects of H_2O_2 will now find expression through peroxovanadates. A profile as important as phosphate transfer is emerging for redox reactions in some of the multiple biological activities of vanadium. The reaction sequences and cycles of vanadate and peroxovanadates integrated into one chart for the first time (figure 21). The pathways fit with most experimental findings, and indicated valid assumptions, and are based on the work from many laboratories.

Acknowledgments

Thanks to many colleagues for the work done in my laboratory: S Vijaya, A S Menon, Meera Rau, Lakshmi Khandke, M S Patole, G Sharada, Vidya Shivaswamy, P Kalyani, H N Ravishankar and Aparna V S Rao. Collaborations with many scientists elsewhere helped our work a great deal: Dr F L Crane, Purdue University, USA, Dr K Namboodiri, Naval Research Laboratory, Washington, USA, Dr V Natarajan, Indiana Univeristy, Indianapolis, USA,Dr Mihir Chaudhuri,North-eastern Hill University, Shillong, Dr B V Venkataraman, St Johns Medical College, Bangalore, and Dr Nashreen Islam, Tezpur Univeristy, Tezpur. I started this work while on a short summer trip to Fred Crane's laboratory in 1979. More than half of the work described here was done since my retirement in 1992, first as CSIR emeritus scientist and then as INSA senior scientist that gave me an extended decade of science involvement. And

Table 4. ***Enzyme systems affected by diperoxovanadate (DPV), inclusive of pervanadate***

Enzyme/system	effect	Reference
Protein tyrosine phosphatase	inhibition	Fantus et al. (1989)
Phospholipase D	activation	Bourgoin & Grinstein (1992) Natarajan et al. (1998)
ERK-1 and ERK-2	activation	Natarajan et al. (1998)
Protein kinase C	down regulation	Natarajan et al. (1998)
Protein tyrosine kinase	stimulation	Hefetz et al. (1990)
Phospholipase C	stimulation	Zick &Eisenberg (1992)
Phosphatidyl inositol-3-kinase	stimulation	Hadari et a. (1992)
Gastric smooth muscle contraction	regulation	Laniyonu et al. (1994)
MAP kinase	activation	Pandey et al.(1995)
NADPH oxidase (neutrophil)	stimulation	Kawakami et al. (1998)
MAP kinase	activation	Krady et al. (1998)
Glyceraldehyde -3-P dehydrogenase	inhibition	Aparna Rao et al. (1998)
Glucose oxidase	inactivation	Aparna Rao et al. (1999)

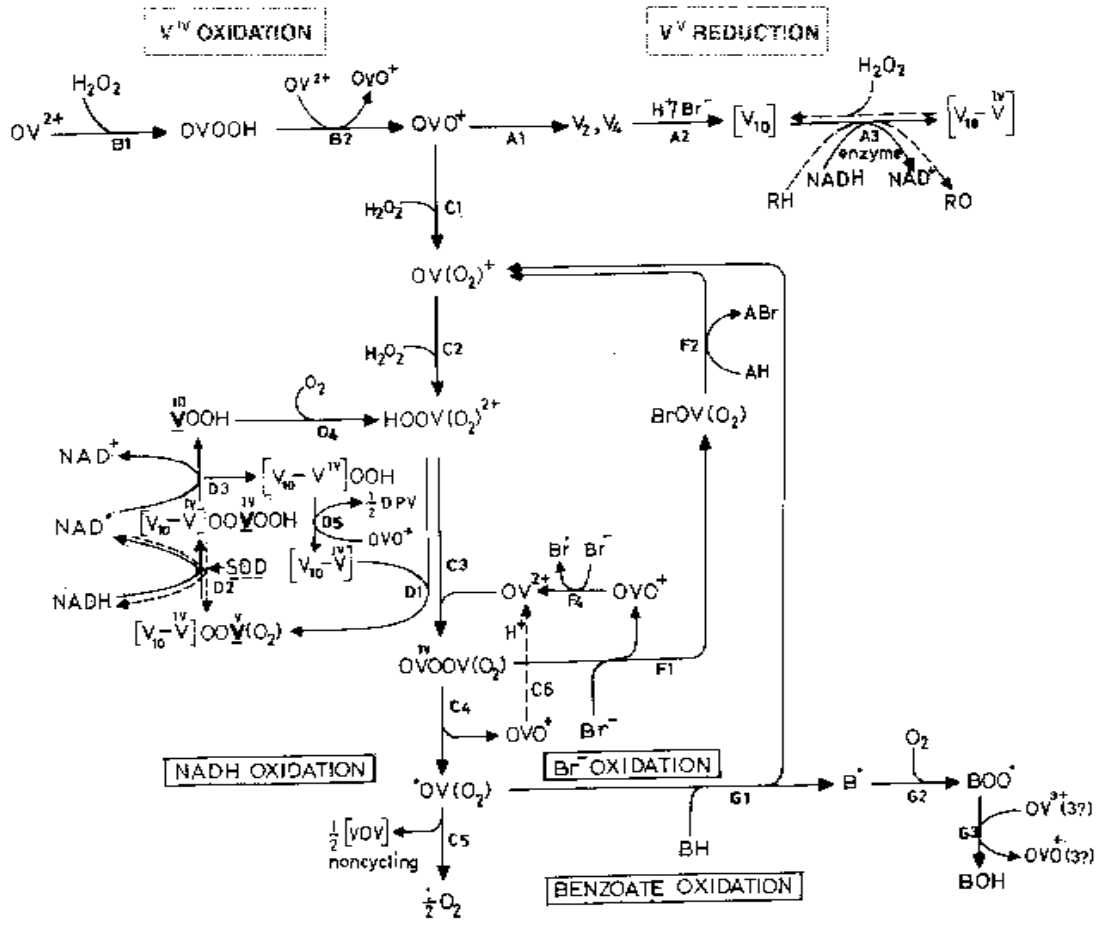

Figure 21. Integrated redox pathways of vanadate and peroxovanadate intermediates. A series: V^{V} reduction; B series: V^{IV} oxidation; C series: backbone pathway of peroxovanadates; D series: NADH oxidation; F series: Br^{-} oxidation; G series: benzoate oxidation. Vanadium valencies other than V^{V} are shown. At least one phosphate anion replaces a +charge on vanadium in most reactions. Cycles shown for oxidation of NADH and Br^{-} fit with peroxovanadate reactions and stoichiometry of reactants. Most likely site of SOD inhibition is reversing D2 (——).

References

Adam-vizi V, Varadi G and Simon P 1981 Reduction of vanadate by ascorbic acid and noradrenaline in synaptosomes; *J. Neurochem.* **36** 1616-1620

Aparna Rao V S, Ravishankar H N and Ramasarma T 1996 Vanadium catalysis in bromoperoxidation reaction; *Arch. Biochem. Biophys.* **334** 121-134

_____, Islam Nashreen S and Ramasarma T 1997 Reactivity of μ-peroxo bridged dimeric vanadate in bromoperoxidation; *Arch. Biochem. Biophys.* **342** 289-297

_____, Ravishankar H N and Ramasarma T 1998 Diperoxo-vanadate participates in peroxidative reactions of H_2O_2 in presence of abundant catalase; *Biochim. Biophys. Acta* **1381** 249-255

_____, Sima P D, Kanofsky J R and Ramasarma T 1999 Inactivation of glucose oxidase by diperoxovanadate-derived oxidants; *Arch. Biochem. Biophys.* **369** 163-173

Aparna Rao V S and Ramasarma T 2000 NADH-dependent decavanadate reductase, an alternative activty of NADP-specific isocitrate dehydrogenase protein; *Biochim. Biophys. Acta.* **1474** 321-330

Bhattacharjee M 1992 Activation of bromide by vanadium pentoxide for the bromination of aromatic hydrocarbons: Reaction mimic for the enzyme bromoperoxidase; *Polyhedron* **11** 2817-2818

_____, Chaudhuri M K, Islam Nashreen S and Paul P C 1990 Synthesis, Characterization and physicochemical properties of peroxo-vanadium (V) complexes with glycine as the hetero-ligand; *Inorg. Chem. Acta* **169** 97-100

_____, Ganguly S and Mukherjee J 1995 Bromination mediated by a vanadium (V)-peroxo complex $[V_2O_2(O_2)_3(GlyH)_2(H_2O)_2]$ (GlyH=Glycine): A functional model for the enzyme bromoperoxidase; *J. Chem. Res.* **5** 80-81

Birskin D P, Thornley W R and Pool R J 1985 Vanadium-dependent NADH oxidation in microsomal membranes of sugar beet; *Arch. Biochem. Biophys.* **236** 228-237

Bourgoin S and Grinstein S 1992 Peroxides of vanadate induce activation of phospholipase D in HL-60 cells; *J. Biol. Chem.* **267** 22908-11916

Brooks H E and Sicilio F 1971 Electron spin resonance kinetic studies of the oxidation of vanadium (IV) by hydrogen peroxide; *Inorg. Chem.* **10** 2530-2534

Butler A, Clague M J and Meister G E 1994 Vanadium peroxide complexes; *Chem. Rev.* **94** 625-638

Cantley L C Jr, Josephson L, Warner R, Yanagisawa N, Laechne C and Guidotti G 1977 Vanadate is a potent (Na, K)-ATPase inhibitor found in ATP derived from muscle; *J. Biol. Chem.* **252** 7421-7423

Carmichael A J 1990 Reaction of vanadyl with hydrogen peroxide, a ESR and spin trapping study; *Free Radical Res. Communs.* **10** 37-45

Casny M, Rehder D, Schmidt H, Vilter H and Conte VA (2000) ^{17}O NMR studyof peroxidase binding to the active centre of bromoperoxidase from *Ascophyllum nidosum; J. Inorg. Chem.* **80** 157-160

Clague M J and Butler A 1995 On the mechanism of cis-dioxovanadium(V)-catalyzed oxidation of bromide by hydrogen peroxide. Evidence for a reactive, binuclear vanadium (V) peroxo complex; *J. Amer. Chem. Soc.* **117** 3475-3484

Commack R 1986 A role for vanadium at last; *Nature (Lond.).* **322** p 312

Crane F L, Mackellar W C, Morre D J, Ramasarma T, Goldenberg H, Grebing C and Low H 1980 Adriamycin affects plasma membrane redox functions; *Biochem. Biophys. Res. Communs.* **93** 746-754

Crans D C 1994 Enzyme interactions with labile oxovanadates and other polyoxometalates; *Comments Inorg. Chem.* **16** 35-76

_____1995 Interactions of vanadate with biogenic ligands; in *Metal ions in Biology* (ed. H Sigel and A Sigel; Publ., Marcel Dekker Inc.) **31** 147-209

Dar D and Fridovich I 1984 Vanadate and molybdate stimulate the oxidation of NADH by superoxide radical; *Arch. Biochem. Biophys.* **232** 562-565

de Boer E, van Kooyk Y, Tromp M G M and Wever R 1986 Bromoperoxidase from *Ascophyllum nodosum*: A novel class of enzymes containing vanadium as a prosthetic group; *Biochim Biophys. Acta* **869** 48-53

de la Rosa R I, Clague M J and Butler A 1992 A functional model of vanadium bromoperoxidase; *J. Am. Chem. Soc.* **114** 760-761

Debaerdemaeker T, Arrietta J M and Amigo J M 1982 Tetrakis (4-ethyl pyridinium) decavanadate; *Acta Cryst.* **838** 2465-2468

Domingo J L, Gomez M, Liobet J M, Corbella J and Keen C L 1991 Improvement of glucose homeostasis by oral vanadyl or vanadate treatment in diabetic rats is accompanied by negative side effects; *Pharmacol. Toxicol.* **68** 249-253

Dubyak G R and Kleinzeller A D 1980 The insulin-mimetic effects of vanadate in isolated rat adipocytes. Dissociation from the effect of vanadate as a (Na^+/K^+) ATPase inhibitor; *J. Biol. Chem.* **255** 5306-5312

Erdmann E, Kraweitz W, Phillip G, Hackbarth I, Schmitz W, Scholz H and Crane F L 1979 Purified cardiac cell membranes with high ($Na^+ + K^+$) ATPase activity contain significant NADH-vanadate reductase activity. *Nature (Lond)* **282** 335-336

Fantus I G, Kodata S, Deregon G, Foster B and Posner B I 1989 Pervanadate [peroxide(s) of vanadate] mimics insulin action in rat adipocytes via activation of the insulin receptor tyrosine kinase; *Biochemistry* **28** 8864-8891

Gadre S R, Bapat S V and Shirsat R N 1992 Molecular electrostatics of $[V_{10}O_{28}]^{6-}$ cluster, a graphics visualization study using PARAM; *Curr. Sci*. **62** 798-801

Hackbarth F, Smith W, Scholtz H, Wetzl H, Erdmann W, Kraweitz W and Phillip G 1980 Stimulatory effect of vanadate on cyclic AMP levels in cat capillary muscle; *Biochem. Pharmacol*. **29** 1429-1432

Hadari Y R, Tzahar E, Nadir O, Rothenberg P, Roberts C T J, LeRoish D, Yerden Y and Zick Y 1992 Insulin and insulinomimetic agent induce activation of phosphatidylinositol-3-kinase upon its association in cell; *J. Biol. Chem*. **267** 17483-17486

Hamstra B J, Colpas G J and Pecoraro V L 1998 Reactivity of diperoxovanadium(V) complexes with hydrogen peroxide: Implications of vanadium haloperoxidase; *Inorg. Chem*. **37** 945-955

Harrison A T and Howarth O W 1985 High-field vanadium-51 and oxygen-17 nuclear magnetic study of peroxovanadates(V); *J. Chem. Soc. Dalton. Trans.* 1173-1177

Heffetz D, Bushkin, I, Dorr, R and Zick, Y 1990 The insulin-mimetic agents H_2O_2 and vanadate stimulate protein tyrosine phosphorylation in intact cells; *J. Biol. Chem*. **265** 2896-2902

Howarth O W, Hunt J R 1979 Peroxo complexes of vanadium (V): A vanadium-51 nuclear magnetic resonance study; *J. Chem. Soc. Dalton. Trans.* 1388-1391

Isupov M N, Dalby A R, Brindley A A, Izumi Y, Tanabe T, Murshudov G N and Littlechild J A 2000 Crystal structure of dodecameric vanadium-dependent bromoperoxidase from red algae *Corallina officinalis*; *J. Mol. Biol*. **299** 1035-1049

Jaswal J S and Tracey A S 1991 Formation and decomposition of peroxovanadium (V) complexes in aqueous solution; *Inorg. Chem*. **30** 3718-3722

Kalyani P and Ramasarma T 1992 Polyvanadate-stimulated NADH oxidation by plasma membranes. The need for a mixture of deca and meta forms of vanadate; *Arch. Biochem. Biophys*. **287** 244-252

_____ and _____ 1993 A novel phenomenon of burst of oxygen uptake during decavanadate-dependent oxidation of NADH; *Mol. Cell. Biochem*. **121** 21-29

Kawakami N, Takemasa H, Yamaguchi T, Hayakawa T, Shimohama S and Fujimoto S 1998 Inhibition of a protein kinase C-independent pathway for NADPH oxidase activation in human neutrophils; *Arch. Biochem. Biophys*. **349** 89-94

Khandke L, Sharada G, Patole M S and Ramasarma T 1986 Vanadate-stimulated NADH oxidation-an intrinsic property of xanthine oxidase; *Arch. Biochem. Biophys*. **244** 742-749

Krady M, Malviya A N and Dupont J 1998 Pervanadate-triggered MAP kinase activation and cell proliferation are not sensitive to PD 98059; *FEBS Lett*. **434** 241-244

Kustin K and Toppen D L 1973 Reduction of vanadium (V) by L-ascorbic acid; *Inorg. Chem*. **12** 1404-1407

Laniyonu A, Saifeddine M, and Hollenberg A S 1994 Regulation of vascular and gastric smooth muscle contractility by pervanadate; *Br. J. Pharmacol*. **113** 403-410

Liochev S and Fridovich I 1986 The vanadate-stimulated oxidation of NAD(P)H by biomembranes is a superoxide-initiated free radical chain reaction; *Arch. Biochem. Biophys.* **250** 139-145

_____ and _____ 1987 The oxidation of NADH by vanadate plus sugars; *Biochim. Biophys. Acta*. **924** 319-322

_____ and _____ 1987b The oxidation of NADH by tetravalent vanadium; *Arch. Biochem. Biophys*. **255** 274-278

_____ and _____ 1990 Vanadate-stimulated oxidation of NAD(P)H in the presence of biological membranes and other sources of O_2^-; *Arch. Biochem. Biophys*. **279** 1-7

Liochev S and Fridovich I 1991 The roles of O_2^-, $^{\bullet}OH$, and secondarily derived radicals in oxidation reactions catalyzed by vanadium salts; *Arch. Biochem. Biophys*. **291** 379-382

Liochev S and Fridovich I 1992 Superoxide generated by glutathione reductase initiates a vanadate-dependent free radical chain oxidation of NADH; *Arch. Biochem. Biophys*. **294** 403-406

Liu Z and Anson F C 2000 Electrochemical properties of vanadium$^{(III,IV,V)}$-salen complexes in acetonitrile. Four electron reduction of O_2 by V^{III}-salen; *Inorg. Chem*. **39** 274-280

Macara I 1980 Vanadium-an element in search of a role; *Trends Biochem. Sci*. **5** 92-94

Macara I G, Kustin K and Cantlely L C Jr 1980 Glutathione reduces cytoplasmic vanadate; mechanism and physiological implications; *Biochim. Biophys. Acta* **629** 95-106

Meera Rau, Patole M S, Vijaya S, Kurup C K R and Ramasarma T 1987 Vanadate-stimulated NADH oxidation in microsomes; *Mol. Cell. Biochem.* **75** 151-159

Menon A S, Meera Rau, Ramasarma T and Crane F L 1980 Vanadate inhibits mevalonate synthesis and activates NADH oxidation in microsomes; *FEBS Lett*. **114** 139-142

Messerschmidt A, Prade L and Wever R 1997 Implications for the catalytic mechanism of the vanadium-containing enzyme chloroperoxidase from fungus *Curvularia inequalis* by X-ray structure of the native and peroxide form; *J. Biol. Chem*. **378** 309-315

Minasi L and Willsky G R 1991 Characterization of vanadate-dependent NADH oxidation stimulated by *Saccharomyces cerevisae* plasma membranes; *J. Bact*. **173** 834-841

Moore R J and Friedl K E 1992 Physiology of nutritional supplements: chromium picolinate and vanadyl sulfate; *Natl. Strength Conditioning Assoc. J.* **14** 47-51

Natarajan V, Vepa S, Shamalal R, Ramasarma T, Ravishankar H N, Scribner W M 1998 Tyrosine kinase and calcium dependent regulation of phospholipase D activation by diperoxovanadate in vascular endothelial cells; *Mol. Cell. Biochem.* **183** 113-124

North P and Post R L 1984 Inhibition of (Na,K)-ATPase by tetravalent vanadium; *J. Biol. Chem.* **259** 4971-4978

Ozaki H and Urokawa N 1980 Effects of vanadate on mechanical responses and sodium-potassium pump in vascular smooth muscle; *Eur. J. Pharmacol.* **68** 339-347

Pandey S K, Chiasson J L and Srivastava A K 1995 Vanadium salts simulate mitogen-activated (MAP) kinases and robosoma S6 kinases; *Mol. Cell. Biochem.* **153** 69-78

Patole M S, Kurup C K R and Ramasarma T 1986 Reduction of vanadate by a microsomal redox system; *Biochem. Biophys. Res. Commun.* **141** 171-175

_____, _____ and _____ 1987 NADH-dependent polyvanadate reduction by microsomes; *Mol. Cell. Biochem.* **75** 161-167

_____, Sharada G and Ramasarma T 1988 Vanadate-stimulated NADH oxidation requires polymeric vanadate, phosphate and superoxide; *Free Rad. Res. Commun.* **4** 201-207

Posner B I, Faure B, Burgess J W, Bevan A P, Lachance D, Zhang-Sun G, Fantus I G, Ng J B, Hall D A, Lum B S and Shaver A 1994 Peroxovanadium compounds. A new class of potent phosphotyrosine phosphatase inhibitors which are insulin mimetics; *J. Biol. Chem.* **269** 4596-4604

Ramasarma T 1990 H_2O_2 has a role in cellular regulation; *Indian J. Biochem. Biophys.* **27** 269-274

_____ and Crane F 1981 Does vanadium play a role in cellular regulations; *Curr. Topics. Cell. Regl.* **20** 247-301

_____ and Ravishankar H N 2003 In quest of new radicals: an oxo-radical of peroxovanadate*; J. Alt. Med.* In press

_____, Mackellar W and Crane F L 1980 Nature of NADH: acceptor oxido-reductase in plasma membranes of mouse liver; *Indian J. Biochem. Biophys.* **17** 163-167

_____, _____ and _____ 1981 Vanadate stimulated NADH oxidation in plasma membranes; *Biochim. Biophys. Acta.* **646** 88-98

Ravishankar H N and Ramasarma T 1993 Multiple reactions in vanadyl-V(IV) oxidation by H_2O_2*; Mol. Cell. Biochem.* **129** 9-29

Ravishankar H N and Ramasarma T 1995 Requirement of a diperoxovanadate-derived intermediate for the interdependent oxidation of vanadyl and NADH; *Arch. Biochem. Biophys.* **316** 319-326

_____, Aparna Rao V S and Ramasarma T 1995 Catalase degrades diperoxovanadate and releases oxygen; *Arch. Biochem. Biophys* **321** 477-484

_____, _____ and _____ 1996 Ethanol-dependent oxygen consumption and acetaldehyde formation during vanadyl oxidation by H_2O_2*; Mol. Cell. Biochem.* **154** 101-106

_____, Chaudhuri M K and Ramasarma T 1994a Oxygen-exchange reactions accompanying oxidation of vanadyl sulfate by diperoxovanadate; *Inorg. Chem.* **33** 3788-3793

_____, Kalyani P and Ramasarma T 1994b NADH oxidation is stimulated by an intermediate formed during vanadyl-H_2O_2 interaction; *Biochim. Biophys. Acta* **1201** 289-297

Rehder D, Holst H, Priebsch W and Vilter H 1991 Vanadate-dependent bromo/iodoperoxidase from *Aspergilus nidosum* also contains unspecific low affinity binding sites for vanadate(V): A ^{51}V NMR investigation, including the model peptides phe-glu and gly-tyr; *J. Inorg. Biochem.* **41** 171-185

Robson R L, Eady R R, Richardson T H, Miller R W, Hawkins M and Postgate J R 1986 The alternative nitrogenase of *Azotobacter chroococcum* is a vanadium enzyme; *Nature (Lond.)* **322** 388-390

Ryan D E, Grant K B, Nakanishi K, Frank P and Hodgon K O 1996 Reactions between vanadium ions and biogenic reductants of unicates: Specroscopic probing for complexation and redox products *in vitro; Biochemsitry* **35** 8651-8661

Sarmah S, and Islam N S 2001 A dinuclear peroxo-vanadium (V) complex with coordinated tripeptide. Synthesis, spectra and reactivity in bromoperoxidation; *J. Chem. Res. (S)* 172-174

_____, Hazarika P, Islam N S, Aparna Rao V S and Ramasarma T 2002 Peroxo-bridged divanadate as selective bromide oxidant in bromoperoxidation; *Mol. Cell. Biochem.* **236** 95-105

Sakurai H and Tsuchiya K 1990 A biomimetic model for vanadium-containing bromoperoxidase; *FEBS Lett.* **260** 109-112

Shechter Y and Karlish S J D 1980 Insulin-like stimulation of glucose oxidation in rat adipocytes by vanadyl ions; *Nature* **284** 556-558

Soedjak H S, Walker J V and Butler A 1995 Inhibition and inactivation of vanadium bromoperoxidase by the substrate hydrogen peroxide and further mechanistic studies; *Biochemistry* **34** 12689-12696

Shi X and Dalal N S 1990 NADPH-dependent flavoenzymes catalyze one-electron reduction of metal ions and molecular oxygen and generates hydroxyl radicals; *FEBS Lett.* **276** 186-191

_____ and _____ 1991 Flavoenzymes reduce vanadium (V) and molecular oxygen and generate hydroxyl radicals; *Arch. Biochem. Biophys*. **289** 355-361

Simon T J B 1979 Vanadate - a new tool for biologists; *Nature (Lond)* **281** 337-338

Stanikiewicz P J, Stern A and Davison A J 1991 Oxidation of NADH by vanadium: kinetics, effects of ligands and role of H_2O_2 and O_2; *Arch. Biochem. Biophys.* **287** 8-17

Svensson I and Stomberg R 1971 Studies on Peroxovanadates 1. Crystal structure of ammonium m-oxo-bis (oxidodiperoxovanadate (V), $(NH_4)_4$ $[OVO(O_2)_2)]$; *Acta. Chem. Scand.* **25** 898-911

Venkataraman B V, Ravishankar H N, Aparna Rao V S, Kalyani P, Sharada G, Namboodiri K, Gabor G and Ramasarma T 1997 Decavanadate possesses a-adrenergic agonist activity and a structural motif common with trans-b form of noradrenaline; *Mol. Cell. Biochem.* **169** 27-36

Vitler H and Rehder D 1987 ^{51}V NMR investigation of a vanadate (V)-dependent peroxidase from *Ascophyllum nodosum* (L) Le; *J. Inorg. Chim. Acta* **136** L7-L10

Vijaya S and Ramasarma T 1984 Vanadate stimulates oxidation of NADH to generate hydrogen peroxide; *J. Inorg. Biochem*. **20** 247-254

Vijaya S, Crane F L and Ramasarma T 1984 A vanadate-stimulated NADH oxidase in erythrocyte membrane generates hydrogen peroxide; *Mol. Cell. Biochem*. **62** 175-185

Vouk V 1979 Vanadium; *in Handbook on the Toxicology of Metals* Ed. L Friber et al. pp 659-674 (Elsevier/ North Holland Biochemical Press)

Vyskocil F, Teisinger J and Dlouha H 1980 A specific enzyme is not necessary for vanadate-induced oxidation of NADH; *Nature (Lond.)* **286** 516-517

Weyand M, Hecht H, Kiess M, Liaud M, Vitler H and Schomburg D 1999 X-ray structure determination of a vanadium-dependent haloperoxidase from *Asophyllum nidosum* at 2.0 Å resolution; *J. Mol. Biol.* **293** 595-611

Yamamoto K, Oyaizu K and Tsuchida E 1996 Catalytic cycle of a divanadium complex with salen ligands in O_2 reduction: Two-electron redox process of the dinuclear center (salen+*N,N'*-ethylenebis (salicylideneamine); *J. Amer. Chem. Soc*. **118** 12665-12672

Yaname H, Fukunaga T, Nigorikawa K, Okamura N and Ishibashi S 1999 Pervanadate activates NADPH oxidase via protein kinase C-independent phosphorylation of p47-phox; *Arch. Biochem. Biophys*. **361** 1-6

Zick Y and Sagi-Eisenberg R 1992 A combination of H_2O_2 and vanadate concomitantly stimulates protein tyrosine phosphorylation and polyphosphoinositide breakdown in different cell lines; *Biochemistry* **29** 10240-10245