Oxygen-Exchange Reactions Accompanying Oxidation of Vanadyl Sulfate by Diperoxovanadate

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The absorption spectrum in the visible range and the ESR spectrum of vanadyl sulfate were lost on addition of diperoxovanadate. The ^{51}V-NMR spectra revealed that diperoxovanadate was reduced to vanadate and its oligomers. With excess vanadyl, tetrameric vanadate was found to be the major product. During this reaction oxygen was released into the medium. The oxygen-release reaction was inhibited by a variety of organic ligands—imidazole, benzoate, formate, mannitol, ethanol, Tris, DMPO, malate, and asparagine. An oxygen-consuming reaction emerged at high concentrations of some of these compounds, e.g. benzoate and ethanol. Using DMPO as the spin-trap, an oxygen-radical species with a 1:2:2:1 type of ESR spectrum was detected in the reaction mixtures resulting from vanadyl oxidation by diperoxovanadate which was unaffected by addition of catalase or ethanol. The results showed that secondary oxygen-exchange reactions occur which depend on and utilize the intermediates in the primary reaction during diperoxovanadate-dependent oxidation of vanadyl sulfate.

Introduction

Vanadium compounds are increasingly being recognized to have biological functions (see for reviews refs 1–3). The antineoplastic activity of vanadate was recognized,[4] and diperoxovanadate in particular was found to increase the life span of mice with murine leukemia.[5] Vanadate, similar to H_2O_2, was found to mimic the action of insulin[6,7] in the uptake and metabolism of glucose in rat adipocytes. Also, the cationic form of tetravalent vanadium, vanadyl (VO^{2+}), and its complexes with chelator compounds were found to be very effective for hypoglycemic action with a possible post-insulin-receptor action.[8] Vanadate and vanadyl compounds on administration to animals were known to be absorbed and distributed in several tissues and their cell components.[9,10] One characteristic of vanadate is the ease with which it oligomerizes to tetramers at neutral pH and to decamers at acid pH at moderately high concentration.[11] Decavanadate ($V_{10}O_{28}^{6-}$) was found to be the active form for reduction[12,13] by a membrane-based enzyme system capable of overall oxidation of NADH[14] and generation of H_2O_2.[15] The purportedly less toxic vanadyl cation was considered to be the storage form[16] and is neither easily oxidized by oxygen[17] nor capable of generating superoxide on auto-oxidation,[18] as often presumed.[19] Oxidation of vanadyl by H_2O_2 was found to be very rapid in acid medium and yielded VO_2^+ and VO_3^+ (monoperoxovanadate). This was accompanied by formation of an oxygen-radical intermediate ($OVOO^{\bullet 2+}$)[20] and some oxygen evolution, both being sensitive to methanol, a scavenger of hydroxyl radicals.[21] Diperoxovanadate was found to be the predominant product at neutral pH in the experiments on oxidation of vanadyl[21,22] and on formation of complexes with metavanadate[23,24] in the presence of excess H_2O_2. Peroxovanadate compounds were found to participate with ease in oxygen-transfer reactions.[25,26] In view of the current interest on the versatile biological functions of diperoxovanadate and vanadyl, we investigated the interaction between these two active vanadium compounds. A tetrameric form of vanadate was found to be the major product on oxidation of vanadyl by diperoxovanadate. With limited vanadyl concentrations, oxygen was released during this reaction. This oxygen-release reaction was inhibited in the presence of a number of organic ligands, and concomitantly an oxygen-consumption reaction emerged.

Experimental Procedures

Chemicals and Solutions. Vanadyl sulfate was obtained from Wilson Laboratories, Bombay, India. Potassium phosphates (mono- and dihydrogen compounds) for making the buffer were obtained from BDH, Bombay, India. Imidazole, benzoic acid, mannitol, tris(hydroxyamino)-methane (Tris), malate, asparagine, dimethylpyrroline *N*-oxide (DMPO), superoxide dismutase (SOD), and catalase were obtained from Sigma Chem. Co., St. Louis, MO.

Potassium diperoxovanadate was prepared by bubbling SO_2 gas through a solution of triperoxovanadate until the solution attained pH 6.0 and turned yellow. Adding ethanol to this solution deposited a yellow microcrystalline product which was filtered out, washed with ethanol,

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and dried. This compound was found to be $K[VO(O_2)_2]H_2O$ by elemental analysis and infrared laser Raman spectroscopic studies. The procedures followed were given in detail in an earlier publication.[27]

The solutions were made in water doubly-distilled in a quartz apparatus. Stock solutions of vanadyl sulfate (35 mM, pH 3.0) and diperoxovanadate (35 mM, pH 7.0) were prepared fresh for each experiment and diluted in water.

Spectroscopy. The visible spectra were recorded in a Hitachi 557 double-beam double wavelength spectrophotometer in 1-cm quartz cuvettes. The two cuvettes were balanced by auto-zeroing through the range of 800 to 400 nm.

The electron spin resonance (ESR) spectra of aqueous solutions of vanadyl sulfate were recorded at room temperature in a capillary in a Varian Model E109 spectrometer under the following conditions: microwave frequency 9.05 GHz, modulation frequency 100 kHz, modulation amplitude 4 × 1 G, time constant 0.064 s, and scan time 4 min. The 8-banded spectra of vanadyl solutions were recorded with field set 3200 G, scan range of 2 × 1000 G, microwave power 2 mW, and a receiver gain of 4×10^3. The recordings of the DMPO adduct of oxygen radicals were done at a field set of 3220 G, scan range of 2 × 100 G, microwave power of 5 mW, and receiver gain of 1.25×10^4.

The ^{51}V-NMR measurements were made at a vanadium frequency of 105.184 883 4 MHz on a Brucker AMX 400 FT spectrometer. Samples were placed in a 10-mm spinning tube with a sealed coaxial tube containing D_2O which provided the lock signal. A spectral width of 62 500 Hz was employed with a pulse width of 20 μs and repetition time of 1.0 s. A line broadening of 25 Hz and an average of 500 scans were applied to the spectra. The chemical shift data of difference forms of pentavalent vanadium are presented as negative values of ppm (δ) (rounded to integers) in the low-frequency direction with reference to $VOCl_3$ at 293 K. The details of the reaction mixtures and conditions used are given in the legend. The spectra represent compounds present in equilibrium at the end of reaction.

Measurement of Oxygen Exchanges. The reaction mixture consisted of phosphate buffer (50 mM, pH 7.0) and diperoxovanadate and vanadyl at specified concentrations. Other compounds where mentioned were added to the buffer before the reactants. The reaction was normally started by adding vanadyl, and the oxygen exchanges were followed until completion of the reaction. All measurements of release and consumption of oxygen were carried out in a reaction vessel of 1.75 mL in a Gilson 5/6H oxygraph, fitted with a Clark electrode, at 30 °C with the stirrer speed set at 6. The recorder pen was set in the middle of the chart paper for experiments wherein both consumption and release were measured. The amount of oxygen released was standardized under these conditions by the increase in dissolved oxygen with excess catalase (0.1 mg/mL) added to a solution containing known amounts of H_2O_2. The reaction vessel was washed with 0.1 N HCl followed by several changes of water whenever catalase was used. The value of 0.224 mM was used for the concentration of dissolved oxygen at 30 °C. The total change in oxygen concentration in the medium was calculated and expressed as mM for easy comparison. A change in oxygen of 0.1 mM corresponds to 175 nmol in the total reaction mixture.

Results

Oxidation of Vanadyl Sulfate by Diperoxovanadate. The absorbance at the peak 780 nm (0.086) of the blue-colored aqueous solution of vanadyl sulfate (5 mM, pH 3.0) decreased progressively on adding batches of diperoxovanadate (5 times each 1 mM final concentration). Another experiment was carried out in 20 mM phosphate buffer at pH 7.0 but at 1 mM vanadyl sulfate, as the solutions became turbid at higher concentrations. In this case the absorbance peak shifted to 600 nm and it disappeared on addition of 1 mM diperoxovanadate (data not shown). Similar loss of the absorbance of vanadyl sulfate solution on addition of H_2O_2 was found earlier in this laboratory. This raised the possibility that the effect of diperoxovanadate may be obtained through release of H_2O_2. The decrease in absorbance in the above experiments with diperoxovanadate was unaffected even in the presence of a high concentration (0.1 mg/mL) of catalase, an enzyme that dismutates H_2O_2 into H_2O and O_2, suggesting that the reaction did not depend on released H_2O_2, if any.

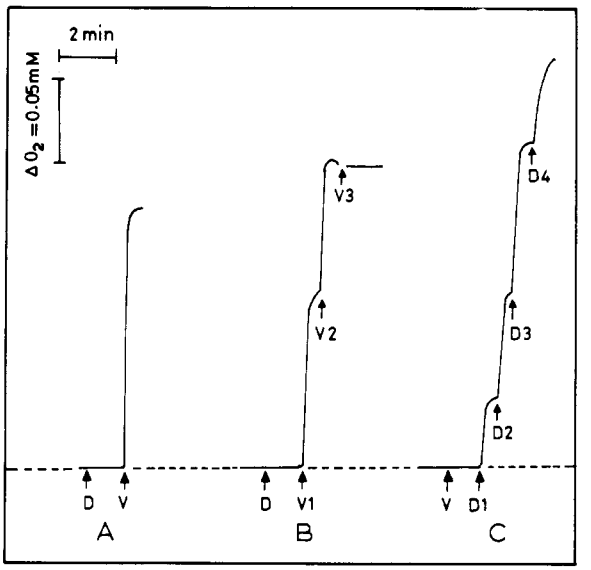

Figure 1. Oxygen release during oxidation of vanadyl sulfate by diperoxovanadate. The reaction was carried out in phosphate buffer (50 mM, pH 7.0) in an oxygraph with the following additions as indicated: (A) D, diperoxovanadate (0.4 mM), and V, vanadyl sulfate (1.0 mM); (B) D, diperoxovanadate (0.4 mM), and V1-V3, vanadyl sulfate (0.2 mM each); (C) V, vanadyl sulfate (1.0 mM), and D1-D4, diperoxovanadate (0.2 mM each).

The oxidative loss of vanadyl on addition of diperoxovanadate was confirmed by ESR spectra. The 8-band spectrum (hyperfine splitting $a = 115$ G), characteristic of tetravalent vanadium (V^{IV}) of an aqueous solution of vanadyl sulfate (pH 2.84), was lost on addition of diperoxovanadate partly at a diperoxovanadate: vanadyl ratio of 1:2 (pH 2.81) and completely at 1:1 (pH 2.14) (data not shown). These experiments were carried out in aqueous solution in the absence of phosphate buffer, as vanadyl sulfate is ESR silent at neutral pH.

Oxygen Release during Interaction of Vanadyl and Diperoxovanadate. An aqueous solution of diperoxovanadate was found to be stable for several hours at neutral pH but degraded slowly in acid medium. A 2 mM solution released oxygen into the medium at the rate of 0.025 mM/min at pH 2.0. Addition of vanadyl sulfate (1.0 mM) to a solution of diperoxovanadate (0.4 mM) in phosphate buffer (50 mM, pH 7.0) resulted in a rapid release of oxygen (0.16 mM total, 1.2 mM/min) into the medium (Figure 1A), and its addition in three batches (each 0.2 mM final concentration) gave values for oxygen release of 0.106 mM, 0.078 mM, and zero (Figure 1B). It is obvious that the reaction stopped because of exhaustion of vanadyl after each of the first two additions. The third addition of vanadyl sulfate elicited no reaction implying exhaustion of diperoxovanadate at a ratio of 1:1 for diperoxovanadate:vanadyl. In another experiment, successive additions of diperoxovanadate (each 0.2 mM final concentration) to a solution of vanadyl sulfate (1.0 mM) released oxygen to the extents of 0.034, 0.064, 0.092, and 0.056 mM. It is noted that the ratios of oxygen released to the reactant added in the above experiments are variable, suggesting simultaneous occurrence of more than one reaction.

The amount of oxygen released was measured at the constant 0.4 mM concentration of vanadyl (Figure 2A) or of diperoxovanadate (Figure 2B) and varying the other reactant. On addition of a specified concentration of the second reactant, a rapid release of oxygen occurred and the reaction terminated as observed in Figure 1. The total amount of oxygen released increased with increase in the concentration of diperoxovanadate and of vanadyl and reached a maximum at ratios of diperoxovanadate:vanadyl of 2:1 and 1:1, respectively (Figure 2C). This experiment was repeated at other constant concentrations of the reactants, and the maximum oxygen release was obtained with the ratios varying

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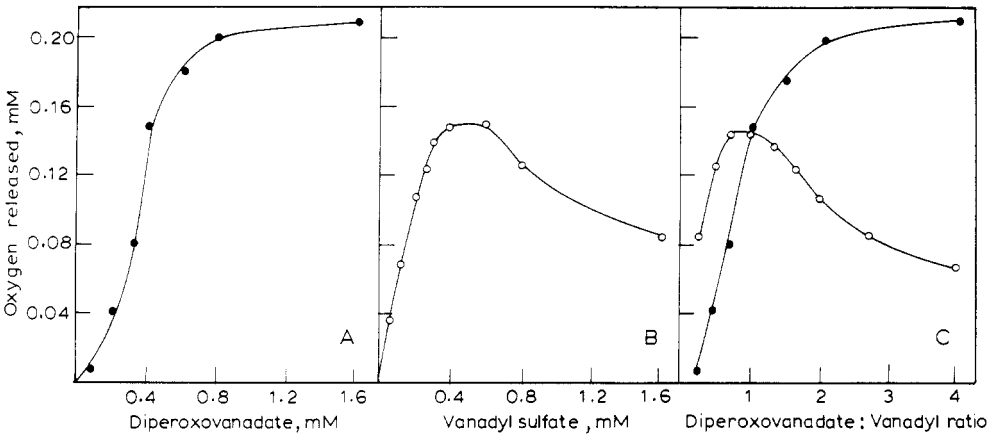

Figure 2. Effect of varying concentrations of diperoxovanadate and vanadyl sulfate on oxygen release. The reaction was carried out in phosphate buffer (50 mM, pH 7.0), and the release of oxygen was measured in an oxygraph with following additions: (A) varying diperoxovanadate concentration with constant 0.4 mM vanadyl sulfate; (B) varying vanadyl sulfate concentration with constant 0.4 mM diperoxovanadate. The data on oxygen release were plotted against the ratio of diperoxovanadate:vanadyl (C).

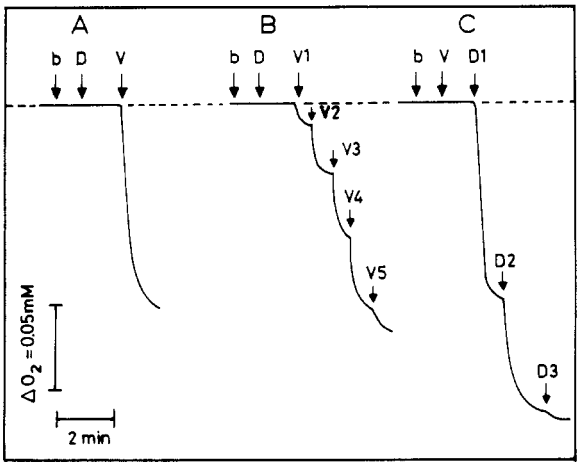

Figure 3. Oxygen-consumption reaction during oxidation of vanadyl sulfate by diperoxovanadate in the presence of benzoate. The reaction was carried out in phosphate buffer (50 mM, pH 7.0) in an oxygraph in the presence of benzoate (b, 1 mM) with the following additions as indicated: (A) D, diperoxovanadate (0.4 mM), and V, vanadyl sulfate (0.4 mM); (B) D, diperoxovanadate (0.4 mM), and V1-V5, vanadyl sulfate (0.2 mM each); (C) V, vanadyl sulfate (0.4 mM), and D1-D3, diperoxovanadate (0.2 mM each).

between 1:1 and 4:1. The maximum oxygen released remained unchanged with diperoxovanadate (Figure 4A) but decreased with vanadyl (Figure 2B) at higher concentrations.

In another set of experiments the effect of catalase (0.05 mg/mL) or SOD (0.01 mg/mL) on the oxygen release reaction was tested by adding a low concentration of diperoxovanadate (0.15 mM) to a solution of vanadyl sulfate (0.1 mM) in phosphate buffer (50 mM, pH 7.0). Catalase had little effect, whereas SOD decreased the rate of the reaction by 50% and the total amount of oxygen released by 25%. At the end of the reaction, it was ascertained that both the added enzymes were active by their specific assays in suitable aliquots of the reaction mixtures.

Oxygen Consumption Reaction in the Presence of Benzoate or Ethanol during Oxidation of Vanadyl by Diperoxovanadate. Inclusion of some quenching compounds of oxygen radicals such as benzoate and ethanol in the reaction mixtures of oxidation of vanadyl by diperoxovanadate showed little effect on the changes in absorbance in the visible spectrum. Testing for their effect on the accompanying oxygen release, it was found that an oxygen consumption reaction emerged instead, as illustrated with benzoate in Figure 3. With 1 mM benzoate present, addition of vanadyl sulfate (1 mM) to a solution of diperoxovanadate (0.4 mM) showed consumption of oxygen at a rate of 0.3 mM/min which terminated after 0.13 mM was consumed (Figure 3A). Five successive additions of vanadyl sulfate (each 0.2 mM final concentration) gave total oxygen consumptions amounting to 0.014, 0.028, 0.039, 0.048, and 0.017 mM (Figure 3B). Similar experiment with three successive additions of diperoxovanadate (each 0.2 mM final concentration) to a solution containing 1 mM each of benzoate and vanadyl sulfate gave decreasing oxygen consumption of 0.118, 0.059, and 0.006 mM (Figure 3C). It is obvious from these results that the oxygen-consumption reaction required the presence of all the three components.

The effect of varying concentration of reactants on the oxygen-consumption reaction was studied using ethanol as the organic ligand. It was found that increasing the concentration of ethanol (A), vanadyl sulfate (B), and diperoxovanadate (C) increased the amount of oxygen consumed (Figure 4). Only with diperoxovanadate the oxygen consumption decreased at higher concentrations after reaching the maximum. Similar results were obtained using benzoate instead of ethanol (data not shown). Maximum oxygen consumption was found when the ratios of diperoxovanadate:vanadyl were in the range 0.1–0.4 (Figure 4D), which is 1 order of magnitude lower than those for the oxygen-release reaction.

The effect of catalase and SOD was tested on this reaction in the presence of benzoate (0.4 mM), vanadyl sulfate (0.4 mM), and diperoxovanadate (0.15 mM) in phosphate buffer (50 mM, pH 7.0). Diperoxovanadate was added last to start the reaction in these experiments to minimize its effect on the enzymes. It was also ensured that the two enzymes were active in aliquots of the reaction mixtures at the end of the reaction. Catalase at a concentration of 0.01 mg/mL fully capable of degrading H_2O_2 was without effect, but at a high concentration of 0.1 mg/mL it decreased the total oxygen consumption by 32% and increased the rate of the reaction by nearly 9-fold. With SOD at 0.01 mg/mL the total oxygen consumption decreased by 27% and the rate by 37%. These effects of catalase and SOD are likely to involve reactions other than dismutation of H_2O_2 and superoxide, respectively.

Decrease in Release and Increase in Consumption of Oxygen in the Presence of Organic Compounds. The above effects of decrease in release and increase in consumption of oxygen are also obtained by a variety of organic compounds besides benzoate and ethanol. The effect of varying the concentrations of a set of organic compounds was tested by adding vanadyl sulfate (0.4 mM) to a solution of diperoxovanadate (0.4 mM) in phosphate buffer (50 mM, pH 7.0). Increasing concentration of each compound showed a progressive decrease in oxygen release. At high concentrations these compounds promoted oxygen consumption to a variable extent. On plotting the concentration of the compound in the log scale (x-axis) and of oxygen exchange in the linear scale (y-axis), a series of straight lines were obtained with crossover from release to consumption of oxygen at a different concentration for each of the compounds (Figure 5): imidazole

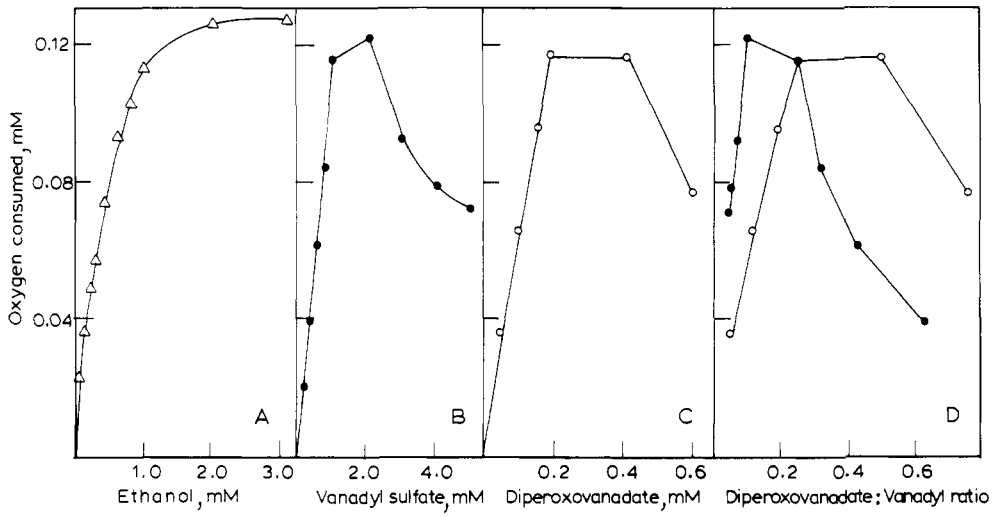

Figure 4. Effect of varying concentrations of ethanol, diperoxovanadate, and vanadyl sulfate on oxygen consumption. The reaction was carried out in phosphate buffer (50 mM, pH 7.0), and the consumption of oxygen was measured in an oxygraph with following additions: (A) varying ethanol concentration with constant 0.8 mM vanadyl sulfate and 0.2 mM diperoxovanadate; (B) varying vanadyl sulfate concentration with constant 1.0 mM ethanol and 0.2 mM diperoxovanadate; (C) varying diperoxovanadate with constant 1.0 mM ethanol and 0.8 mM vanadyl sulfate. The data on oxygen consumption were plotted against the ratio of diperoxovanadate:vanadyl (D).

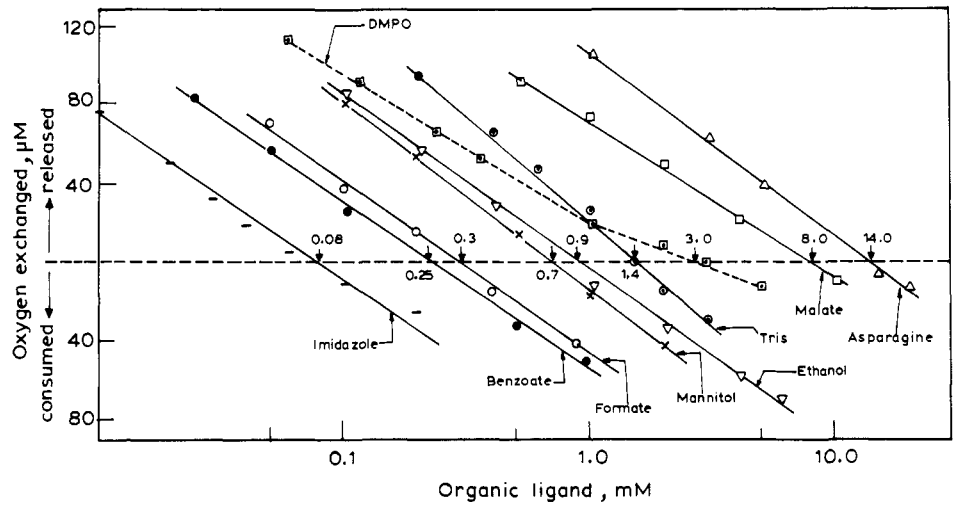

Figure 5. Effect of organic ligands on the oxygen-exchange reactions. The reaction mixtures consisted of phosphate buffer (50 mM, pH 7.0), diperoxovanadate (0.4 mM), vanadyl sulfate (0.4 mM), and increasing amounts of the organic compounds—imidazole, benzoate, formate, mannitol, ethanol, Tris, DMPO, malate, and asparagine. The reaction was started in each case by adding vanadyl, and the change in oxygen concentration was measured in an oxygraph. The total amount of oxygen exchanged on termination of the reaction was plotted on the *y*-axis against the concentration of the organic compounds on the *x*-axis (log scale).

(0.08 mM), benzoate (0.25 mM), formate (0.3 mM), mannitol (0.7 mM), ethanol (0.9 mM), Tris (1.6 mM), DMPO (3 mM), malate (8 mM), and asparagine (14 mM). Aspartate, histidine, and tartarate were also effective (data not given). These compounds, include hydroxyl radical scavengers (benzoate, formate, mannitol, ethanol, Tris), a spin trap for oxygen radicals (DMPO), organic acids (malate, citrate, tartarate), amino acids (asparagine, aspartate), and singlet oxygen quenchers (imidazole, histidine). Of these compounds, those that quench reactive oxygen species were most effective in promoting the oxygen-consumption reaction. The crossover concentration was found to be lower at higher concentrations of the reactants, and at other proportions of the reactants it became ill-defined as oxygen consumption emerged before complete suppression of oxygen release (data not shown). Thus the two reactions seem to be independent and can also occur together, and in all such cases the faster consumption reaction appeared first followed by the release. Since sufficient dissolved oxygen was available in the medium, the oxygen-consumption reaction need not depend on the released oxygen. An apparent inverse relationship exists between the two reactions.

Identification of the Products of Interaction of Diperoxovanadate and Vanadyl by ^{51}V-NMR Spectra. It was obvious from the foregoing studies that vanadyl was oxidized by the peroxo groups of diperoxovanadate. It can be expected that both the vanadium compounds will be converted at neutral pH to orthovanadate and its oligomers. This was tested by studying ^{51}V-NMR spectra of solutions in phosphate buffer (50 mM, pH 7.0) containing different concentrations of diperoxovanadate and vanadyl. The spectra, recorded 5 min after mixing the components, represent an equilibrium mixture derived from the products of the reaction (Figure 6).

A concentration of 10 mM was used for recording the spectrum in line 1 which showed the major peak corresponding to diperoxovanadate (–707 ppm), two minor peaks of triperoxovanadate (–731 ppm), and a dimer of diperoxovanadate (–754 ppm), as well as trace amounts of other vanadate species (–500 to –600 ppm region). At a diperoxovanadate:vanadyl ratio of 2:1 (line 2), the peaks of peroxovanadates decreased and three new peaks appeared corresponding to dimeric vanadate (–571 ppm), cyclic tetravanadate (–578 ppm), and monoperoxovanadate (–619 ppm). On increase of the concentration of vanadyl to a ratio of diperoxovanadate:vanadyl of 1:1 (line 3), the peaks corresponding to peroxovanadates disappeared. It is interesting to note that this condition is characterized by the complete loss of added vanadyl and the maximum release of oxygen. The products present under this condition are the dimer (–572 ppm) and the

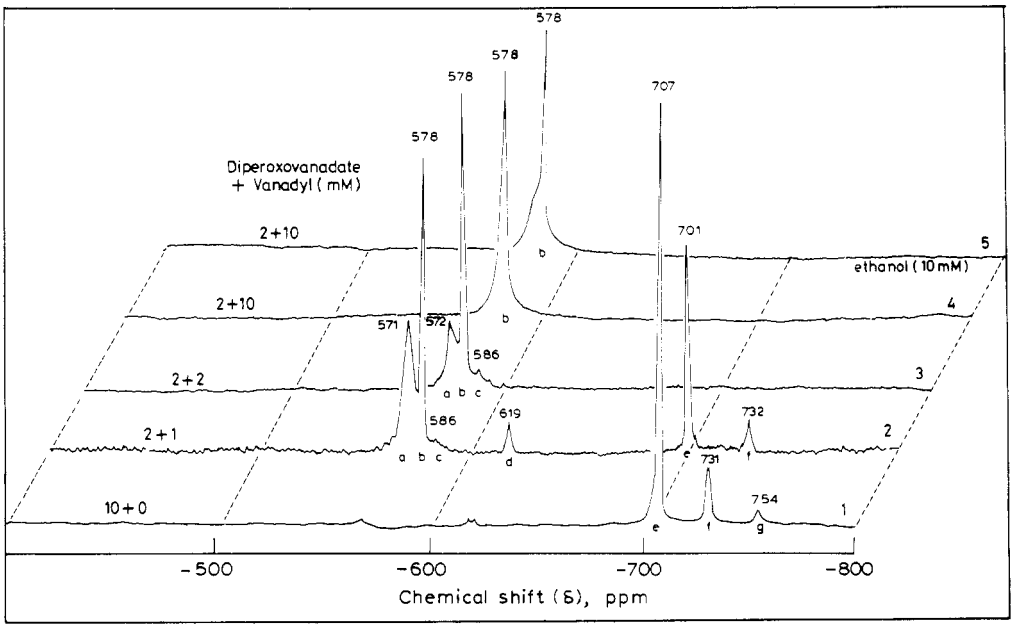

Figure 6. Identification of the products formed on interaction between vanadyl sulfate and diperoxovanadate by ^{51}V-NMR spectra. The reaction mixture consisted of phosphate buffer (50 mM, pH 7.0), diperoxovanadate (10 mM for line 1 and 2 mM for lines 2–5), and vanadyl sulfate (1, 2, 10, and 10 mM for lines 2–5, respectively). In addition, 10 mM ethanol was included in the experiment given in line 5. The spectra were recorded 5 min after mixing by which time the reaction was complete. The vanadium oxidation products were identified by their chemical shifts (δ): (a) dimer of orthovanadate (–570 to –572 ppm), (b, c) cyclic form of tetrameric vanadate (–578 ppm), (d) monoperoxovanadate (–619 ppm), (e) diperoxovanadate (–701 and –707 ppm), (f) triperoxovanadate (–731 and –732 ppm), and (g) dimer of diperoxovanadate (–754 ppm). The peaks in the figures were marked by the small alphabet letters at the bottom and the number of –δ at the top.

tetramer (–578 ppm) of vanadate. It is possible that these represent composite signals of phosphovanadate complexes.

In presence of a 5-fold excess of vanadyl without (line 4) and with ethanol (line 5) only the peak at –578 ppm appeared. This result is reproducible, and only a shoulder at –572 ppm was seen in some experiments. Neither ethanol at this concentration nor the high concentration of vanadyl seems to affect the product. The final product with or without the organic ligand gave a single peak at –578 ppm at this ratio of diperoxovanadate:vanadyl of 0.2:1, which also gave maximal ethanol-dependent oxygen consumption (Figure 3D). It is indeed surprising that a single major product was obtained under these conditions in view of the known rapid exchange between oligomers of vanadate. We considered the possibility that this product is a vanadate–phosphate complex, described by Gresser et al.[29] Only one peak appeared in the reaction mixtures with (–578.88 ppm) and without (–577.92 ppm) phosphate buffer (50 mM, pH 7.0). A close examination of the data of Gresser et al.[29] revealed that the phosphate-induced change in the chemical shift is limited to orthovanadate (–551 ppm peak shifted to –558 ppm when 52 mM phosphate was added) but not others including the tetramer whose peak at –575 ppm remained unchanged. The –578 ppm compound, thus, is unlikely to be a phosphovanadate complex. Identification of the above compounds was based on the assignments given by Howarth and Hunt,[23] Gresser et al.,[29] and Jaswal and Tracey.[24] The major (–574 or –575 ppm) peak obtained with metavanadate solutions was identified as the cyclic form of vanadate tetramer.[29-32] Small differences in the chemical shift values occur due to variable protonation. In the present experiments the product derived from diperoxovanadate and vanadyl giving a peak at –578 ppm is considered to be the cyclic tetramer of vanadate.

Formation of Oxygen Radical Species during Oxidation of Vanadyl by Diperoxovanadate. The primary reaction of oxidation of vanadyl and reduction of diperoxovanadate leading to the product of tetrameric vanadate is accompanied by an oxygen release reaction. A similar reaction of oxygen release and formation of a peroxovanadium oxygen radical species ($OVOO^{\bullet 2+}$) during H_2O_2-dependent oxidation of vanadyl had been documented by Brooks and Sicilio.[21] Both the oxygen release and the radical species were abolished in the presence of methanol, and this led to the suggestion that dismutation of two molecules of the oxygen radical species give raise to VO_3^+ and VO^{3+} and release of a molecule of dioxygen. Formation of oxygen radical species in the present experiments was therefore tested using the spin trap DMPO. The hyperfine splitting of the DMPO adduct of oxygen radicals with a 1:2:2:1 quartet ($a_N = a_H = 14.9$ G) was obtained in the ESR spectrum of a solution containing phosphate buffer (20 mM, pH 7.0), DMPO (100 mM), vanadyl sulfate (1 mM), and diperoxovanadate (1 mM) (data not shown). The spectrum remained unchanged on addition of ethanol at a concentration of 10 mM and decreased in peak heights at 170 mM. The split of the spectrum into triplets of doublets expected of $^{\bullet}OH$ radicals was not observed. Catalase added at a concentration of 10 μg/mL, sufficient to degrade any H_2O_2 formed, did not affect the spectrum and at 100 μg/mL decreased peak heights but did not abolish the spectrum. SOD (10 μg/mL) was found to be without any effect, but no conclusion can be drawn because the enzyme was found to be inactivated by diperoxovanadate. The lack of effect of catalase and ethanol on the ESR spectrum indicated that the oxygen radical species responsible for this DMPO adduct is not an $^{\bullet}OH$ species and is likely to be an $^{\bullet}OV$ species, as all such adducts yield similar ESR spectra.[34] The ESR spectrum was not observed when a high proportion of vanadyl was used, as under this condition the radical species would be expected to be further reduced to vanadate and its tetramer.

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Formation of *p*-Hydroxybenzoate from Benzoate during Oxidation of Vanadyl by Diperoxovanadate. A hydroxylation or epoxidation of the organic ligand can also account for the oxygen-consumption reaction. This was tested using benzoate. The reaction mixture (1.75 mL) consisted of a phosphate buffer (50 mM, pH 7.0), benzoate (1 mM), diperoxovanadate (0.2 mM), and vanadyl sulfate (0.8 mM). After incubation for 10 min at 30 °C, the mixture was acidified and extracted with ethyl ether and the phenolic acids in the extract were separated by paper chromatography as described earlier.[35] A single spot corresponding to *p*-hydroxybenzoate was found. The reaction was also carried out in an oxygraph for measuring oxygen consumption, and on its termination an aliquot was used for measuring *p*-hydroxybenzoate by the method of Bray et al.[36] The mean ± standard deviation values of six separate experiments were as follows: 101 ± 3 μM oxygen consumed and 79 ± 11 μM *p*-hydroxybenzoate. It was clear that, in the case of benzoate, part of the oxygen consumed was used for producing the hydroxylated product and the balance for oxidation of vanadyl.

Discussion

The foregoing experiments describe for the first time interaction between diperoxovanadate and vanadyl, two of the biologically active molecules of vanadium. Multiple reactions are known to occur on oxidation of vanadyl by H_2O_2 with the preferred end product being monoperoxovanadate in acid medium[21] and diperoxovanadate at neutral pH.[22] We now find that similar reactions occur on substituting H_2O_2 with diperoxovanadate. The overall reaction in this case involves both oxidation of vanadyl and the reduction of diperoxovanadate leading to their conversion to vanadate and its oligomers.

The loss of vanadyl-dependent spectra on addition of equimolar concentrations of diperoxovanadate indicated oxidation of vanadyl. The peroxo groups of diperoxovanadate are apparently reduced by vanadyl, a strong reducing agent. This is probably facilitated by forming an addition complex, followed by internal redox rearrangement and transfer of one oxygen to OV^{2+} to form OVO^+, a characteristic of vanadyl oxidation according to Brooks and Sicilio.[21] The oligomerization of the monomer species of vanadate is a well-known process[28,32] with the monomer (V_1), dimer (V_2), and cyclic and linear forms of tetramer (V_4) existing in an equilibrium, determined by the concentration and pH. Evidence is provided for the presence of V_2 and V_4, but not for any phosphovanadate complex, in the reaction mixtures with phosphate buffer (50 mM, pH 7.0) based on their chemical shifts in ^{51}V-NMR spectra. The formation of the cyclic tetramer of vanadate as the major product on oxidation of vanadyl by diperoxovanadate constitutes the primary reaction.

It is realized that the primary reaction will have a sequence of steps and intermediates, albeit proof for their identity is meager. An oxygen-radical species is clearly indicated as one such intermediate. This is based on the earlier proposal[21] that the breakdown of the μ-peroxo bridge of the complex of vanadyl-H_2O_2 leads to an •OH radical. A parallel reaction with diperoxovanadate instead of H_2O_2 yields an oxygen-radical species, $^{\bullet}OV(O_2)^{2+}$. This is inferred by the appearance in the reaction mixtures with low vanadyl concentrations of the ESR spectrum of the DMPO adduct (1:2:2:1 quartet) that remained unaffected by catalase or ethanol, in contrast to that of H_2O_2.[33] Proof on the identity of this radical species is required.

Extensive evidence is provided in the present studies on the occurrence of oxygen release as a secondary reaction. Both oxygen release and formation of the oxygen-radical species require small concentrations of vanadyl but are suppressed at high concentrations. Thus the instability of this radical species may be employed in the intramolecular electron rearrangement to release dioxygen by a dismutation reaction, as already proposed for $OVOO^{\bullet 2+}$, a related oxygen-radical species[20,21] formed under acidic conditions. One of the products of such a dismutation reaction will be monoperoxovanadate, and this is identified in the NMR spectrum at low proportion of vanadyl favorable to oxygen release. Implicating the oxygen-radical species also offers explanation for the dependence of oxygen release on the occurrence of the primary reaction.

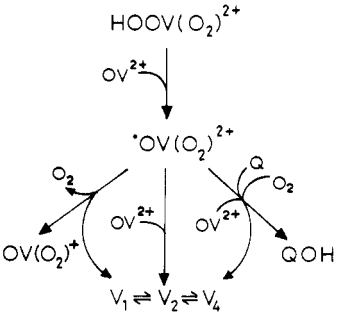

Figure 7. Schematic representation of the reactions involved in oxidation of vanadyl by diperoxovanadate. The primary reaction is oxidation of vanadyl (OV^{2+}) by diperoxovanadate [$HOOV(O_2)^{2+}$] with vanadate oligomers (V_1, V_2, V_4) in equilibrium as the products derived from both reactants. An oxygen-radical species [$^{\bullet}OV(O_2)^{2+}$], whose presence is indicated by an ethanol-stable ESR spectrum (1:2:2:1 quartet) of the DMPO adduct, is shown as an intermediate. Two secondary reactions of oxygen exchange are shown to arise from this intermediate—an oxygen release by dismutation to monoperoxovanadate and vanadate and an oxygen consumption in the presence of organic ligands (Q) to form QOH and V_4. No attempt is made to show exact stoichiometry of the reactions.

The oxygen-consumption reaction is complex and requires both the vanadium compounds and their reaction in the presence of an organic ligand (Q) which may form a complex with a vanadium species. The oxygen-radical species, $^{\bullet}OV(O_2)^{2+}$, is the likely vanadium species for such a complex as oxygen consumption emerged on suppressing oxygen release.

More data are required to identify the product(s) of reduction of oxygen. A ratio of about 1:4:1 of diperoxovanadate:vanadyl:oxygen was observed for oxygen consumption which provides sufficient vanadyl to reduce oxygen to H_2O_2 in addition to its reaction with diperoxovanadate. But free H_2O_2 was not present in the medium. This was indicated by the lack of release of oxygen on adding excess catalase after termination of oxygen consumption (data not given). But any H_2O_2 formed would be consumed for oxidation of vanadyl to vanadate. The need for vanadyl and its loss during oxygen consumption offer indirect support for this. Detection of *p*-hydroxybenzoate as a product when benzoate was used indicated that hydroxylation is also a possible reaction in the case of some ligands. The ability of peroxo complexes of vanadium for supporting epoxidation and hydroxylation reactions is already known.[25] The oxygen consumption reaction is shown to include formation of both QOH and vanadate tetramer as products and needs further investigation for clarity of the subsystems involved.

The results and their evaluation in this paper give an insight, but not complete explanation, of the multiple reactions that occur between vanadyl and diperoxovanadate as schematically shown in Figure 7. We realize the lacunae in the identity of the proposed oxygen-radical intermediate of peroxovanadate and its reactivity with vanadyl as well as organic ligands. These competing reactions which produce reactive molecules are likely to have a crucial role in the biological activity of these vanadium compounds.

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