ANNALS OF CLINICAL AND LABORATORY SCIENCE, Vol. 27, No. 6

Vanadium (IV) Formation in the Reduction of Vanadate by Glutathione Reductase/NADPH and the Role of Molecular Oxygen*

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ABSTRACT

Experimental evidence documenting the formation of a relatively stable V(IV) species appears to be important with regard to the biochemical mechanism of reduction of vanadate by enzymatic systems. The present study demonstrates that a mixture of vanadate and glutathione reductase/nicotinamide-adenine-dinucleotide phosphate (NADPH), in phosphate (pH 7.2) buffer generates V(IV) under ambient conditions. Once formed, V(IV) does not rapidly autoxidize so as to defy detection by electron spin resonance (ESR) spectroscopy. The aerobic environment was guaranteed by preparing reaction mixtures in well stirred, wide mouth, standard test tubes in air over a period of 50 minutes, and by making ESR measurements in nuclear magnetic resonance (NMR) sample tubes as well as oxygen-permeable Teflon tubes. The V(IV) ESR signal intensity was found to increase linearly with time elapsed after reaction initiation. The linear growth of the V(IV) species also shows that this species is fairly stable, over a period of at least 50 minutes. Similar V(IV) stability data were obtained from $VOSO_4$, a model compound as a source of V(IV). The results obtained in the present study demonstrated that V(IV) can be generated in the reduction of V(IV) by glutathione reductase in the presence of NADPH under aerobic condition.

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0091-7370/97/1100-0422 $01.50

Introduction

Although vanadium, a transition metal widely distributed in nature, is an essential nutrient for plants, animals and microorganisms,[1] epidemiological studies have suggested a correlation between exposure to vanadium-containing particles and the incidence of human cancers.[2,3] It enhances mutagenesis and sister chromatid.[4] Vanadium also functions as a modulator of cellular regulatory cascades and oncogene expression.[5] While the mechanism of its carcinogenic action is unclear, it is known that reactive oxygen species play a role in tumorigenesis, specially at the tumor promotion stage.[6,7] Reactive oxygen species are able to induce expression of several oncogenes, for example, c-fos and c-myc.[8] Previous studies[9,10] have shown that reduction of vanadate by certain flavoenzymes, such as glutathione reductase, generates V(IV) in the presence of NAD(P)H. During the reduction process, molecular oxygen was reduced to H_2O_2, which reacts with V(IV) to generate hydroxyl radical via Fenton-like reaction. The generation of oxygen radicals was suggested to be one of the mechanisms by which vanadium exerts its cellular effects.

Since the biological activation of vanadium and its related free radical generation depend on the oxidation state of vanadium, the study of cellular reduction of V(V) is required. While our earlier studies[9,10] have demonstrated that reduction of vanadate generates V(IV), the role of molecular oxygen in the mechanism of V(IV) generation remains controversial.[11] The possible reaction of V(IV) with molecular oxygen may affect the stability of V(IV) and its ability to generate oxygen radical in cellular systems. The present study was undertaken to investigate whether or not aerobic oxygen plays any role in the V(IV) formation in the reduction of vanadate by glutathione reductase in the presence of NADPH.

Materials and Methods

Phosphate buffer (pH 7.2, containing 2 mM ethylenediamine tetraacetic acid [EDTA])* and $VOSO_4$ (as a source of V(IV)),† sodium vanadate ($NaVO_3$) (as a source of V(V)),† glutathione reductase (GSSG-R) from bovine intestinal mucosa,† and NADPH† were purchased from leading companies, as was Chelex-100 chelating resin.‡ The phosphate buffer was treated with Chelex-100 to remove possible transition metal ion contaminants.

Measurements of ESR were made with a Varian E4 ESR spectrometer and a flat cell assembly. For better aeration, certain measurements were carried out using an oxygen-permeable Teflon tubing as described in earlier studies.[12,13] Briefly, the samples were drawn into an oxygen-permeable Teflon tube§ (0.623 mm i.d.; wall thickness, 0.03 mm). This tube was inserted into a quartz ESR tube open at both ends. During the measurements, air was blown around the sample without having to disturb the alignment of the tube within the ESR cavity.

Oxygen consumption rates were measured in two independent ways, using (a) a Clark oxygen electrode (model 5300)‖ and (b) the technique of ESR oximetry.[14] The ESR oximetry method is based on the interaction between nitroxide free radicals and molecular oxygen, which broadens the nitroxide ESR signals via Heisenberg spin-exchange in a concentration-dependent manner. The advantage of this method includes rapid response time and sensitivity over a broad range of oxygen concentrations. The paramagnetic probe used in this study was 4-oxo-2,2,6,6-tetramethylpiperidine-d_{16}-1-^{15}N-1-oxyl. The sample was drawn into a glass capillary tube which was then sealed at both ends and placed in the ESR tube. The ESR spectra were recorded every minute. The oxygen dependence of the probe's ESR spectrum was quantitated by changes in the spectral superhyperfine structure, whose values were related to specific oxygen concentrations. This methodology enabled essentially parallel ESR measurements of oxygen concentration

* Fisher Scientific, Pittsburgh, PA.

† Sigma Chemical Co., St. Louis, MO.

‡ Bio-Rad.

§ Zeus Industrial Products, Raritan, NJ.

‖ Yellow Springs Instrument Co., Yellow Spring, OH.

and V(IV) formation for any given sample composition.

Results and Discussion

Detection of V(IV) Using a Nuclear Magnetic Resonance (NMR) Tube

In figure 1 is shown a typical ESR spectrum obtained from a fraction of a drop taken out of a mixture of 2.5 mM vanadate, 15 units/ml glutathione reductase, and 2.5 mM NADPH in a phosphate buffer solution loaded into a 5 mm o.d. NMR tube. The mixture was kept in air and well stirred for 50 minutes in a standard test tube before taking a fraction of a drop for the ESR measurement (figure 1). The spectrum consists of eight peaks, which is characteristic of V(IV), as discussed earlier.[9,10,15] This result implies that a long-lived V(IV) species is indeed formed, under aerobic conditions, in a phosphate solution containing vanadate, glutathione, and NADPH. It should be noted that the V(IV) ESR spectrum obtained using the NMR tube (figure 1) is not very strong because of the dielectric loss associated with the use of an aqueous sample in a 5 mm o.d. NMR sample tube.

The time dependence of the V(IV) ESR signal intensity has also been measured using both a flat cell and a 5 mm o.d. NMR tube. In figure 2, line (a), is shown the time course obtained from a mixture containing vanadate and glutathione reductase/NADPH in pH 7.2 phosphate buffer. These reactants were mixed in a test tube and were transferred immediately to a flat cell. The ESR measurements were made at various time intervals while the sample remained in the flat cell. It may be

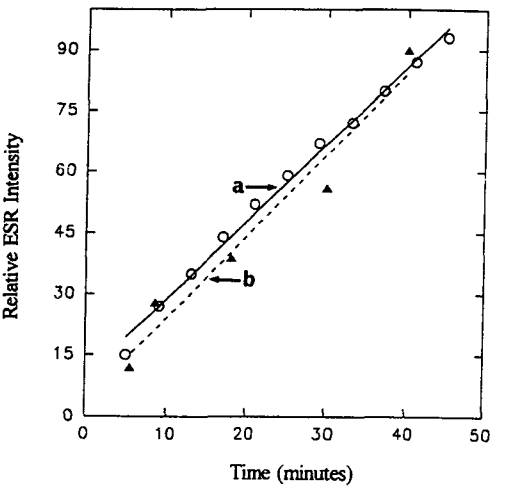


FIGURE 2. (a) Time course of V(IV) ESR signal from a mixture of 2.5 mM $NaVO_3$, 2.5 mM NADPH and 15 units/ml glutathione reductase in phosphate buffer (pH 7.2). The mixture was transferred to a flat cell immediately after the reactants were mixed in a test tube. The sample remained in the flat cell throughout the experiment. (b) Time course of V(IV) ESR signal from the mixture in (a). The reactants were stirred in a test tube for various time intervals and transferred to a flat cell just before each measurement.

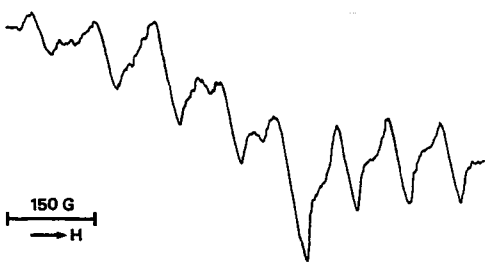


FIGURE 1. (a) ESR spectrum recorded 52 minutes after mixing in a phosphate buffer solution (pH 7.2) of 2.5 mM $NaVO_3$, 2.5 mM NADPH and 15 units/ml glutathione reductase. The reductants were stirred in a test tube under ambient air for 52 minutes and then a small droplet was transferred to a 5 mm o.d. NMR sample tube for the ESR measurement.

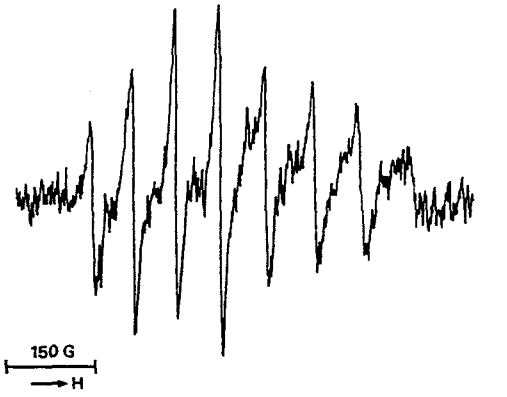


FIGURE 3. ESR spectrum obtained from a mixture of 2.5 mM $NaVO_3$, 2.5 mM NADPH and 15 units/ml glutathione reductase in phosphate buffer (pH 7.2). The mixture was made in a test tube and then transferred into an oxygen-permeable Teflon tubing. The quartz tube was continuously perfused with a flow of air. Spectrum recorded 20 minutes after mixing the reactants.

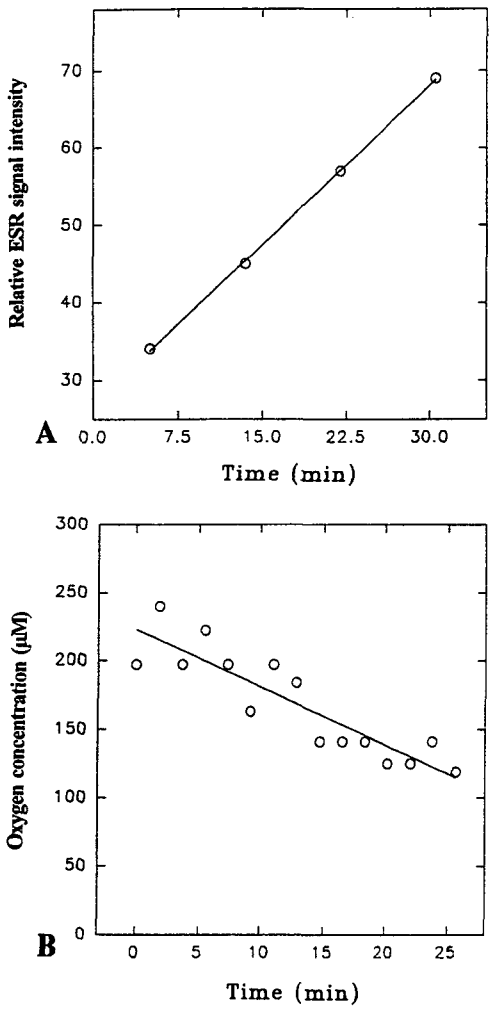


FIGURE 4. (a) Time course of V(IV) ESR signal measured from a mixture of 2.5 mM $NaVO_3$, 2.0 mM NADPH and 7.5 units/ml glutathione reductase in phosphate buffer (pH 7.2). The sample was transferred to an oxygen-permeable Teflon tubing immediately after the reactants were mixed in a test tube. (b) Oxygen consumption rate of a mixture of 2.5 mM $NaVO_3$, 2.0 mM NADPH and 7.5 units/ml glutathione reductase in phosphate buffer (pH 7.2). The mixture was drawn into glass capillary tube that was sealed and placed inside quartz ESR sample tube. Other experimental conditions are described in the Materials and Methods section.

noted that the V(IV) ESR signal intensity increases linearly with time. A similar time course was obtained from a separate experiment in which the sample was stirred every 2 minutes in a test tube under ambient air and transferred to a flat cell for ESR measurements at various times. Again, a straight line was obtained (figure 2, line (b)). The similarity of the plots in figures 2(a) and 2(b) implies that the flat cell itself does not cause any artifact as long as the reactants are well mixed under aerobic atmosphere.

DETECTION OF V(IV) USING AN OXYGEN-PERMEABLE TEFLON TUBE

In another effort to obtain an ESR spectrum from the vanadate/glutathione reductase/NADPH mixture under aerobic environment, an oxygen-permeable Teflon tube was utilized following the procedure described earlier by Samuni et al.[12] and Glockner et al.[13] In figure 3 is shown a typical spectrum obtained from a phosphate buffer solution of vanadate, glutathione reductase, and NADPH utilizing this technique. The reactants were mixed in a test tube under ambient air and then transferred to the oxygen-permeable Teflon tubing which was inserted into a quartz tube open at both ends. The sample was perfused with air during the entire course of the ESR measurement. The observation of the V(IV) signal under these conditions (figure 3) clearly shows that the absence of oxygen is not a prerequisite for the formation of V(IV) in this vanadate/glutathione reductase/NADPH mixture.

OXYGEN CONSUMPTION AND V(IV) FORMATION

As another test of the relationship of oxygen activity to the mechanism of V(IV) generation,

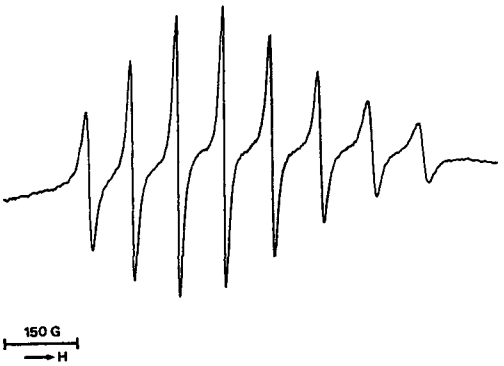
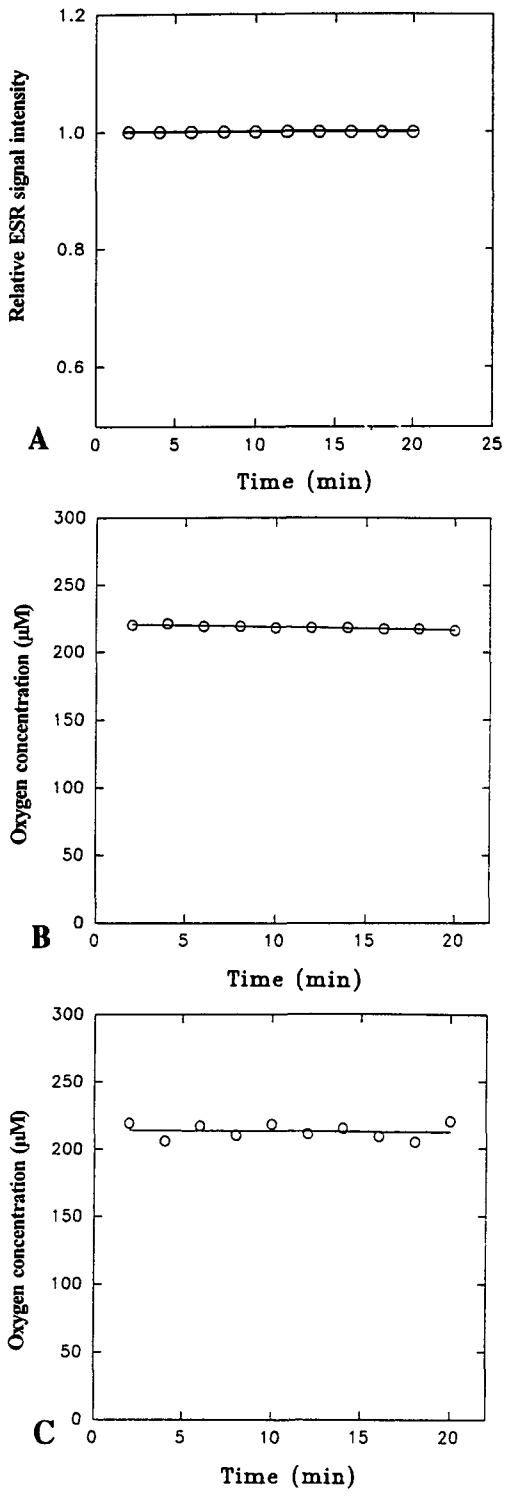


FIGURE 5. Typical ESR spectrum from a phosphate buffer solution (pH 7.2) containing 2 mM $VOSO_4$ in a gas permeable Teflon tube.



the time course of V(IV) generation was measured under well aerated conditions utilizing the previously described oxygen-permeable Teflon tubing. Again, as shown in figure 4(a), the V(IV) signal increased linearly with time. To probe the role of oxygen in the generation of V(IV), a freshly made sample for oxygen consumption measurement was used. These measurements utilized a glass capillary tube sealed at both ends. It may be noted from figure 4(b) that while the change in oxygen consumption for the sample in a sealed glass capillary does take place, the effect is much smaller compared to the increase in V(IV) concentration for the sample in an oxygen-permeable tubing. In a 20 minute period, for example, while the amount of oxygen decreases by about 50 percent, the amount of V(IV) increases by about 200 percent. It is noted that the small amount of oxygen consumed is in agreement with our suggestion[9] that during the flavoenzyme-catalyzed reduction of vanadate, molecular oxygen was simultaneously reduced to H_2O_2 via O_2^- as an intermediate. The H_2O_2 thus formed reacts with the freshly generated V(IV) to generate $^{\cdot}OH$ radical, as demonstrated by ESR spin trapping.[9,10] These data thus support our claim that dissolved oxygen plays a minor, if any, role in the mechanism of reduction of vanadate by glutathione reductase/NADPH.

Parallel Measurements of V(IV) Autoxidation and Oxygen Consumption

In order to examine whether or not a V(IV) species rapidly autoxidizes under aerobic conditions,[12] ESR measurements were carried out on the stability of V(IV) and any accompanying oxygen consumption using $VOSO_4$ as a model

FIGURE 6. (a) Time dependence of ESR signal intensity of 2 mM $VOSO_4$ in a phosphate buffer (pH 7.2). (b) Oxygen consumption rate of a 2 mM $VOSO_4$ in a phosphate buffer solution (pH 7.2) measured by a Clark oxygen electrode. (c) Oxygen consumption rate of 2 mM $VOSO_4$ in a phosphate buffer solution (pH 7.2) as measured by ESR oximetry methodology as described under Materials and Methods.

compound. In figure 5 is shown the ESR spectrum obtained from a pH 7.2 phosphate buffer solution of $VOSO_4$ in an oxygen-permeable Teflon tubing. In figure 6(a) is shown the time dependence of the V(IV) signal intensity, whereas in figures 6(b) and 6(c) are shown the time dependence of the oxygen concentration, as measured by a Clark electrode and ESR oximetry, respectively. It can be noted that V(IV) does not undergo any significant degree of autoxidation over a period of 20 minutes, and, in fact, over many hours (data not shown).

This study was undertaken to demonstrate the following important points: (1) a mixture of vanadate and glutathione reductase/NADPH in phosphate (pH 7.2) buffer, under ambient conditions, does generate V(IV), and (2) once formed, V(IV) does not rapidly autoxidize so as to defy detection by ESR spectroscopy.

As demonstrated in figures 1 and 3, this study documents that a long-lived V(IV) species is formed under aerobic conditions. The aerobic environment was guaranteed by preparing reaction mixtures in well stirred, wide mouth, standard test tubes in air over a period of 50 minutes and by making ESR measurements in NMR sample tubes as well as oxygen-permeable Teflon tubes. The V(IV) ESR signal intensity was found to increase linearly with time elapsed after reaction initiation. If the O_2-dependent chain reactions were the key step for the generation of V(IV), this would not be so. The linear growth of the V(IV) species also shows that this species is fairly stable, over a period of at least 50 minutes.

Similar V(IV) stability data were obtained from $VOSO_4$, a model compound as a source of V(IV) (figure 6). Thus, the results obtained in the present study demonstrated that V(IV) can be generated in the reduction of V(V) by glutathione reductase in the presence of NADPH under aerobic condition.

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