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Oxidation of hydroxyurea with oxovanadium(V) ions in acidic aqueous solution

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Abstract

Hydroxyurea (HU) effectively reduces vanadium(V) into vanadium(IV) species (hereafter V^V and V^{IV} species, respectively) in acidic aqueous solution via the formation of a transient complex followed by an electron transfer process that includes the formation and subsequent fading out of a free radical, U· (U· ≡ H_2N–C(=O)N(H)O·). The electron paramagnetic resonance (EPR) spectra of U· in H_2O/D_2O solutions suggest that the unpaired electron is located predominantly on the hydroxamate hydroxyl-oxygen atom. Visible and V^{IV}–EPR spectroscopic data reveal HU as a two-electron donor, whereas formation of U·, which reduces a second V^V, indicates that electron transfer occurs in two successive one-electron steps. At the molarity ratio $[V^V]/[HU] = 2$, the studied reaction can be formulated as: $2\ V^V + HU \rightarrow 2\ V^{IV} + 0.98\ CO_2 + 0.44\ N_2O + 1.1\ NH_3 + 0.1\ NH_2OH$. Lack of evidence for the formation of NO is suggested to be a consequence of the slow oxidation of HNO due to the too low reduction potential of the V^V/V^{IV} couple under the experimental conditions used.

The nuclear magnetic resonance (^{51}V-NMR) spectral data indicate protonation of $(H_2O)_4V^VO_2^+$, and the protonation equilibrium constant was determined to be $K = 0.7\ M^{-1}$. Spectrophotometric titration data for the V^V–HU system reveal formation of $(H_2O)_2V^VO(OH)U^+$ and $(H_2O)_3V^VOU^{2+}$. Their stability constants were calculated as $K_{110} = 5\ M^{-1}$ and $K_{111} = 22\ M^{-2}$, where the subscript digits refer to $(H_2O)_4V^VO_2^+$, HU and H^+, respectively.

Keywords: Dioxovanadium(V); Hydroxyurea; Redox; Mechanism; EPR spectroscopy

1. Introduction

The pharmacology of hydroxyurea (HU) has drawn the attention of many scientists. HU is an S-phase specific inhibitor of ribonucleotide reductase with a broad spectrum of anti-tumor effects [1]. It has been reported that HU effectively decreases blast cell count and resolves leukemic infiltration of lungs in patients with acute myeloblastic leukemia [2], arrests progression of unresectable or recurrent benign meningiomas [3], and among others significantly improves clinical outcomes in patients with sickle cell disease [4,5]. Nowadays, hydroxyurea represents a new treatment for sickle cell anemia.

The main benefit from the treatment of patients with sickle cell anemia with HU arises from an increased production of fetal hemoglobin that prevents the polymerization of deoxy sickle cell hemoglobin. However, some patients appear to benefit from HU even before the production of fetal hemoglobin is increased, pointing to other mechanisms that can account for the HU activity.

It was reported that HU, as many other hydroxamic acids, also acts as a nitric oxide donor under oxidative conditions in vitro [6,7], and that direct nitric oxide producing reactions of HU and hemoglobin, myoglobin, or hemin may contribute to the overall pathophysiological properties of this drug [8]. Chemically, the treatment of hydroxyurea with hydrogen peroxide and copper(II) sulfate produces a

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"NO-like" species capable of *N*-nitrosating morpholine [9]. Investigation of the NO generation by H_2O_2-dependent peroxidation of hydroxyurea in the presence of copper-containing proteins such as Cu, Zn-superoxide dismutase (Cu, Zn-SOD) or ceruloplasmin as a catalyst, indicates that NO release from hydroxyurea might be mediated by $HO^{\bullet}$ derived from the copper catalyzed Fenton-like reaction [10]. Oral administration of HU in the treatment of sickle cell disease produced in vivo detectable nitrosyl hemoglobin [11–13].

On the other hand, investigation of inhibitory effects of nitro-vasodilators and HU on DNA synthesis in cultured human aortic smooth muscle cells, indicates that NO does not mediate the inhibitory action of HU in this system [14]. These few examples demonstrate the diversity of the mechanisms of HU action which may account for its therapeutic activity, whereas its NO unit structure gave rise to a serious question whether HU takes effect via an NO mechanism? Therefore, clear molecular mechanisms describing the actions of HU remain to be established [15,16].

In order to postulate such molecular mechanisms it is desirable to broaden the general knowledge on the reactivity of HU by studying its oxidation by various oxidants. Since under certain conditions the pathophysiological activities of HU are evidently associated with the production of NO, and for some other activities the presence of this compound is not required, it seems interesting to clarify at the molecular level what factors may play a critical role in the formation of NO during oxidation of HU. One of the factors that may play a major role could be the reduction potential of the involved oxidizing agent. Therefore, in this paper we present our results on equilibrium studies of the reaction between HU and oxovanadium(V) ions in acidic aqueous solution.

Aside from its physiological relevance [17,18], a main benefit from using oxovanadium(V) as oxidizing agent arises from its pH dependent reduction potential in acidic aqueous solution due to the presence of coordinated oxo-ions [19,20].

Although, due to the high acidity used throughout the experiments, the herein reported results may have no direct relevance for physiologically important conditions, the observation of the same free radical formed during the oxidation of HU either with vanadium(V) in highly acidic medium, or with hexacyanoferrate(III) in neutral aqueous medium, indicates a certain physiological relevance. Our results may certainly serve as a valuable basis for a better understanding of the behavior of HU in solution, and eventually, its therapeutic effectiveness.

2. Experimental

2.1. Materials

All water used was deionized and then twice distilled in an all-glass apparatus, first from an alkaline solution of $KMnO_4$. Hydroxyurea, $NaClO_4$ and $NaVO_3$ were reagent grade from Sigma and were used without further purification. A 0.76 M stock solution of vanadium(V) was prepared by dissolving the appropriate amount of $NaVO_3$ in hot water and filtered through a Millipore filter after cooling. The concentration of V^V was determined by titration with $FeSO_4(NH_4)(SO_4)$. A stock solution of $NaClO_4$ was prepared from anhydrous $NaClO_4$ standardized by passage through a DOWEX 50W-X8 strong acid cation exchange column in the H^+ form, and titrated against standard NaOH. In all measurements, a solution of $NaVO_3$ was mixed with $HClO_4$ of appropriate concentration and left for a few hours to stabilize (allowing transformation of the tetrahedral into octahedral vanadium(V) species) prior to mixing with HU. An ionic strength of 2 M was maintained in solution by addition of appropriate amounts of a $NaClO_4$ stock solution.

The amount of CO_2 produced during the oxidation of HU with vanadium(V) was determined by titration of the excess $Ba(OH)_2$ after a gas stream through the sample was passed through a standard solution of $Ba(OH)_2$ at 25 °C. Ammonia was determined via the indophenole formation reaction, by the standard analytical procedure [21–23], whereas hydroxylamine was determined with *p*-nitrobenzaldehyde [24]. The reaction solutions were tested for nitrite and nitrate as the reaction products by the reactions with sulfanilic acid and naphthilamine [25–27], and by ion-exchange chromatography, respectively. The results indicate that these two species were not formed under our experimental conditions.

2.2. Physical measurements

Rapid-scan studies were performed on an Applied Photophysics stopped-flow spectrophotometer equipped with a J&M (Aalen, Germany) diode array detector. The nuclear magnetic resonance (^{51}V-NMR) spectra were recorded on a Bruker AVANCE DRX 400 WB spectrometer equipped with a super conducting BC-94/89 magnet system. The ^{51}V-signal shift was recorded relative to an internal standard ($VOCl_3$ in a sealed capillary). The electron paramagnetic resonance (EPR) spectra were recorded at room temperature on a Varian E-109 EPR spectrometer. The spectra were recorded immediately following complex-formation caused by the mixing of V^V with HU (in both aqueous and heavy-water solutions) at a frequency of 9.36 GHz with a microwave power of 10 mW, modulation amplitudes of 2.5 G (0.25 mT) and a frequency of 100 kHz. The infra red (IR) spectra of gaseous products were recorded on a Mattson Instruments, Research Series FT-IR, the mass spectra were recorded on a Shimadzu GC–MS QP 5050A spectrometer, and gas chromatograms were recorded on an AutoSystem gas chromatograph (Perkin-Elmer, Norwalk, USA) equipped with split/splitless injector and thermal conductivity detector. Turbochrom software was used for raw data analysis. Detector temperature was set to 120 °C and injector temperature was set to 300 °C. Injection volume was 100 μL with a split ratio of

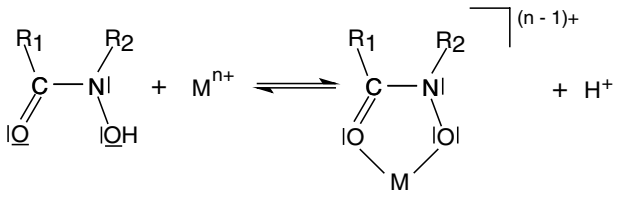
1:10. CP-PoraPLOT Q capillary column, 25 m × 0.53 mm (Varian, Palo Alto, USA) was used in all experiments and column oven temperature was 27 °C. Helium was used as a carrier gas at a pressure of 20 kPa.

3. Results

Immediately upon mixing of aqueous acidic solutions of $V^{V}O_2^+$ (hereafter $V^{V}O_2^+$ represents $(H_2O)_4V^{V}O_2^+$) and HU, within a few milliseconds mixing-time, a red-violet color forms, which slowly fades afterwards. The initial color formation is too fast to be measured by stopped-flow technique, but the color disappears on the stopped-flow time-scale. Oxovanadium(IV) is the only light-absorbing species at 760 nm, and zero-absorbance at that wavelength indicates that in the initial color-formation stage no reduced vanadium ion is formed. Consequently, the formation of color must exclusively be related to the chelation of oxovanadium(V) ions by HU, analogously to the chelation of desferrioxamine B [28]. On the other hand, both the visible (760 nm) and EPR spectral changes confirmed the formation of V^{IV} during the slow, i.e. kinetically measurable, stage. During the same time, formation of a free radical was also observed by EPR spectroscopy. Therefore, the observed disappearance of the red-violet color definitely corresponds to the subsequent redox reaction. It should be noted that in the absence of V^{V}, hydroxyurea was found to be stable during that time.

4. Complex-formation reactions

Complex-formation equilibria were studied by spectrophotometric titration, measuring the visible spectra of solutions immediately after mixing the reactants when no more than 3% (typically less than 1%) of the formed color disappeared. An increase in the metal ion, ligand, or proton concentration, caused an increase in absorbance (Table S1, Supporting Information). The latter dependence indicates a preferential complex-formation at higher acidity, which is in contrast to the expected release of one proton during complex-formation, according to the following general equation (Scheme 1), wherein for hydroxyurea $R_1 \equiv NH_2$ and $R_2 \equiv H$. Therefore, our results suggest that during the complexation of the $V^{V}O_2^+$ ion with HU, two protons per vanadium are bound. One of the protons may come from HU, but another must come from the solvent molecule. It can be concluded that the protonation of at least one oxo ligand of the chelated and/or unchelated oxovanadium(V) species must occur during complex-formation.



Scheme 1.

In order to check the possible protonation of unchelated VO_2^+ ion within the employed pH range, the effect of acid on the ^{51}V-NMR spectra of dioxovanadium(V) ions was investigated in the absence of HU in the solution. Addition of perchloric acid to aqueous solutions of VO_2^+ ion caused a slight shift of the vanadium resonance line toward higher frequencies (Fig. 1), indicating protonation of VO_2^+ species in the studied proton concentration range (0.1–3.0 M $HClO_4$).

The value of the equilibrium constant for reaction (1), $K = 0.7(2)\ M^{-1}$ (hereafter the number in parentheses represents a single standard deviation of the reported parameter expressed in terms of the last reported significant digit), corresponding to $pK_a = -0.15$ for $V^{V}(OH)O^{2+}$ (hereafter $V^{V}(OH)O^{2+}$ represent $(H_2O)_4V^{V}(OH)O^{2+}$), was calculated by fitting Eq. (2) to the observed experimental values of Δv_{obs}. In Eq. (2) the symbols $\Delta v_{VO_2^+}$ and $\Delta v_{VO(OH)^{2+}}$ represent the chemical shifts of the dioxovanadium ion and its protonated form, respectively, which were calculated to differ by 1.3 ppm.

$$\mathbf{VO_2^+} + \mathbf{H^+} \overset{K}{\rightleftarrows} \mathbf{VO(OH)^{2+}} \quad (1)$$

$$\Delta v_{obs} = \frac{\Delta v_{VO_2^+} + \Delta v_{VO(OH)^{2+}} K[\mathbf{H^+}]}{1 + K[\mathbf{H^+}]} \quad (2)$$

In all later computations of equilibrium constants for the formation of the V^{V}–HU complexes, the calculated value of $K = 0.7\ M^{-1}$ was used as a fixed parameter.

Fig. 2 shows the observed UV–visible spectra recorded immediately after mixing V^{V} and HU solutions at various acidities. An increase in the proton concentration causes the same bathochromic spectral shift no matter which of the reactants, i.e. V^{V} or HU, is present in excess, indicating that mono HU–V^{V} complexes are formed predominantly. The higher the solution acidity, the larger the apparent stability constant that could be calculated indicating that complex-formation is accompanied by the binding of a proton. Therefore, in acidic solutions two forms of the

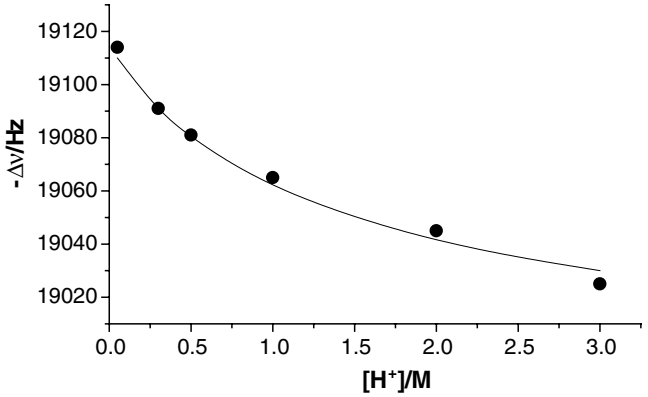


Fig. 1. The proton dependence of the ^{51}V-NMR chemical shift. Conditions: $[V^{V}] = 10$ mM, $\theta = 25$ °C, $I = 3.0$ M (H/$NaClO_4$). The theoretical line was calculated according to Eq. (2).

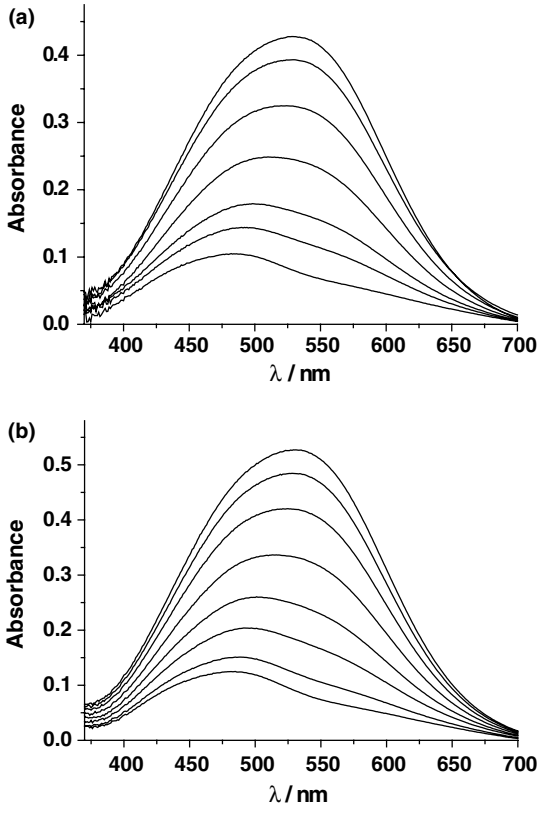


Fig. 2. Spectra recorded immediately after mixing of the reactants. (a) [HU] = 0.5 mM, [V^V] = 15 mM, I = 2, [H^+] = 0.1 (bottom), 0.2, 0.3, 0.5, 0.7, 0.9, 1.0 M (top) 25 °C. (b) [V^V] = 0.5 mM, [HU] = 20 mM, [H^+] = 0.05 (bottom), 0.1 0.2, 0.3, 0.5, 0.7, 0.9, 1.0 M (top), I = 2, 25 °C.

mono(hydroxyurea)vanadium(V) complex exist, which are rapidly equilibrated through the binding/release of at least one proton.

Taking into account that coordination of metal ions by monohydroxamic acids requires release of one proton per coordinated molecule [29], the first considered reaction model was $V^VO_2^+ + HU \rightleftarrows V^VO_2U + H^+$, to which a complexation reaction with no *proton release* ($V^VO_2^+ + HU \rightleftarrows V^VO(OH)U^+$) was added (hereafter V^VO_2U, $V^VO(OH)U^+$ and V^VOU^{2+} represent $(H_2O)_2V^VO_2U$, $(H_2O)_2V^VO(OH)U^+$ and $(H_2O)_3V^VOU^{2+}$, respectively). This model was rejected because, as expected, it completely failed to accommodate the mentioned acidity effect, and the data-fit did not converge. The second model considered included a complexation reaction in which no *protolysis* occurs, and to which a proton binding reaction was added.

$$V^VO_2^+ + HU \overset{K_0}{\rightleftarrows} V^VO(OH)U^+$$

$$V^VO_2^+ + HU + H^+ \overset{K_1}{\rightleftarrows} V^VOU^{2+} + H_2O$$

Fitting this model to 87 data points resulted in a convergence with an acceptable χ^2-value (Fig. S1). The iterated parameters were calculated as $K_0 = [VO(OH)U^+][VO_2^+]^{-1}[HU]^{-1} = 5(2)\ M^{-1}$ and $K_1 = [VOU^{2+}][VO_2^+]^{-1}[HU]^{-1}[H^+]^{-1} = 22(6)\ M^{-2}$, from which $pK_a = 0.632$ can be calculated for the reaction $VOU^{2+} \rightleftarrows VO(OH)U^+ + H^+$.

5. Stoichiometry of the redox reaction

The redox reaction was slow enough to be followed by rapid-scan stopped-flow or, under certain experimental conditions, by conventional UV–Vis spectrophotometry, to observe the fade in color of the complexes formed in the fast pre-equilibrium stage. The reaction was "irreversible" under the experimental conditions used, in a way that no color was observed after 10 half-lives of the reaction.

The stoichiometric coefficients of V^V and HU for the redox reaction were determined by measuring absorbances of reaction solutions at 760 nm, where only $V^{IV}O^{2+}$ absorbs considerably. Independently of the $HClO_4$ concentration (within the range 0.2–1.0 M), the leveling-off of the observed absorbance for 0.02 M HU was found at 0.04 M $V^VO_2^+$ (Fig. S2), indicating that HU acts as a two-electron donor in the reduction of $V^VO_2^+$ to $V^{IV}O^{2+}$ ions. The reaction stoichiometry was confirmed by measuring the V^{IV}–EPR line intensities in 1 M $HClO_4$ (Fig. S3), since no increase in the intensity of the V^{IV}–EPR lines was found above a molar ratio of HU:V^V = 1:2. These two figures clearly indicate that the oxidation products of HU are not capable of reducing the third V^V to V^{IV}. Expressed in another way, V^V ions in acidic solution can not oxidize hydroxyurea to a NO product in which the oxidation state of nitrogen is +1, i.e. the third electron could not be taken away from an HU molecule by VO_2^+ ions. Based on these results, the reactant stoichiometric coefficients could be defined as: $\mathbf{2}V^VO_2^+ + \mathbf{1}HU \rightarrow 2V^{IV}O^{2+}$ + other products.

In order to identify the other products and to determine their stoichiometric coefficients, gas evolved during the redox reaction was analyzed by mass and FTIR spectroscopy, gas chromatography, as well as by chemical analysis. After passing the gaseous products through a solution of $Ba(OH)_2$, the titration of the excess $Ba(OH)_2$ revealed 0.98 moles of CO_2 produced per mole of HU. The presence of CO_2 in the gaseous products accounts for the medium-strong peaks observed at 2361 cm^{-1} and around 620 cm^{-1} in the IR spectrum (Fig. S4), whereas the peaks at 2245, 2204, 1301 and 1272 cm^{-1} could be assigned to N_2O(g). The signal observed at 44 m/z^+ in the mass spectrum confirms the formation of these two gasses. The formation of 0.44 moles of N_2O per mole of HU was determined by gas chromatographic analysis of the gas evolved from 4 ml of the reaction solution containing 0.10 M V^V, 0.05 M HU and 0.2 M $HClO_4$ in a 20 ml vial.

The formation of NO could not be proven, either directly through the IR spectrum of the gaseous products, and by measurements with a NO-sensitive electrode in the reaction solution, or indirectly, via formation of nitrite or nitrate ions which were not detected by specific chemical reactions in solution. However, 1.1 moles of ammonia and 0.1 mole of hydroxylamine formed per mole of HU were determined in the reaction solution containing 0.04 M V^V, 0.02 M HU and 0.02 M $HClO_4$.

From the obtained results the studied reaction can be formulated as:

$$2V^{V} + NH_2CONHOH \rightarrow 2V^{IV} + 0.98\ CO_2 + 0.46\ N_2O + 1.1\ NH_3 + 0.1\ NH_2OH$$

The determined reaction stoichiometry indicates that the reaction that accounts for the decomposition of HU in acidic aqueous solutions involves its oxidation with V^{V} ions, whereas the contribution of V^{V}-catalyzed hydrolysis is almost negligible.

6. Free radical characterization

The formation and subsequent fading of the free radical signal was monitored by recording EPR spectra of reaction solutions on the time scale of the redox reaction. The EPR signal of the radical consists of six resonance lines and is placed between the fourth and fifth resonance lines of the well known octet of the $V^{IV}O^{2+}$ ion (Fig. 3).

The resolved six resonance line spectrum of the radical ($g = 2.0067$, Fig. 4a) is brought about by coupling of the unpaired electron with the ^{14}N nucleus ($a_N = 0.8$ T), giving rise to triplet resonance lines. Further splitting into doublets is caused by proton coupling ($a_H = 1.2$ mT). No further splitting, or extensive broadening of signals indicates deprotonation of the hydroxamate hydroxyl group and dissociation of paramagnetic V^{IV} from the complex with an hydroxyurea free-radical, respectively. The observed result is in excellent agreement with the Lassman and Liermann EPR measurements of a free radical produced by the oxidation of HU with Cu(II) and H_2O_2 ($a_N = 0.8$ T, $a_H = 1.16$ mT) [30]. However, contrary to their placement of the unpaired electron into the nitrogen atomic orbital, we prefer distribution of the unpaired electron density predominantly in an sp^3-like orbital of the hydroxylate-oxygen atom. If the electron density was mainly located on the nitrogen atom, a stronger coupling with ^{14}N-nucleus and a smaller g-value value would be expected [31,32]. Moreover, the free-radical ligand would probably not be deprotonated.

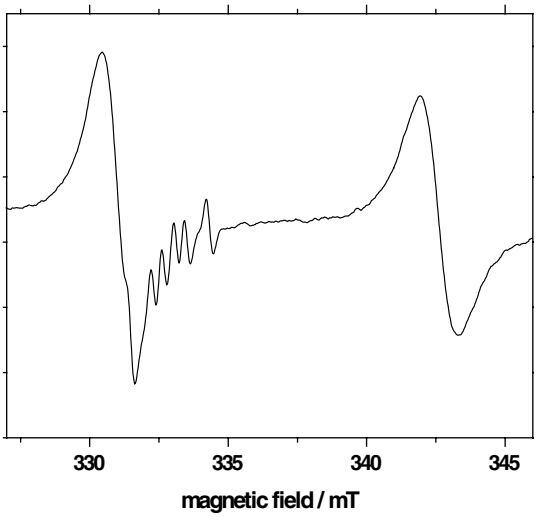


Fig. 3. The six resonance lines of the free radical ($g = 2.0067$) in the second-derivative EPR-spectrum of a solution of V^{V} and HU, recorded at room temperature and frequency of 9.36 GHz with a microwave power of 10 mW. Condition: $[HU] = [V^{V}] = 1$ mM, $I = 2$, $[H^+] = 0.1$ M, $\theta = 25$ °C.

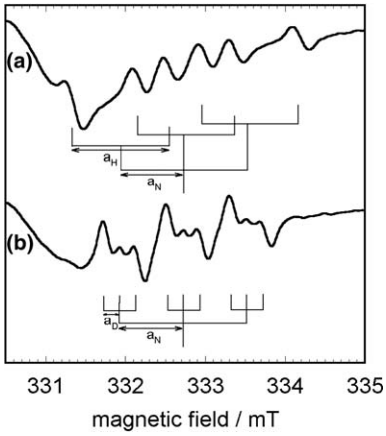


Fig. 4. The resolved EPR spectrum of the radical formed in the reaction of V^{V} with HU, recorded at 9.36 GHz with 10 mW microwave power. The bars beneath the spectrum indicate the triplet due to nitrogen ^{14}N hyperfine interaction ($a_N = 0.8$ mT), which further split into doublets ($a_H = 1.2$ mT) in the non-deuterated system (a), and into triplets ($a_D = 0.2$ mT) in the partly (94 %) deuterated system (b). $[HU] = [V^{V}] = 1$ mM, $I = 2$, $[H^+] = 0.1$ M, $\theta = 25$ °C.

The proposed assignment further confirms the recorded EPR spectra in a partially deuterated (94%) reaction system. As expected, on displacing N-H by N-D, the EPR spectrum changed from a triplet of doublets into a triplet of triplets with about six times smaller deuterium atom couplings (Fig. 4b). All these findings confirm formation of the same nitroxide free radical ($U^{\cdot} \equiv H_2N$–C(=O)N(H)O$^{\cdot}$) by oxidation of HU with excess of either hydrogen peroxide, copper(II) sulfate, tyrosyl radical, oxyhemoglobin [30,33,34], or dioxovanadium(V) ions.

7. Discussion

The postulated reaction mechanism for the oxidation of various ligands by V^{V} in acidic aqueous medium consists of the formation of a transient species followed by an electron transfer process [35–39]. The spectral data presented in Fig. 2 undoubtedly confirm the formation of a colored transient species, the mono(hydroxyureato)vanadium(V) complex, similarly to the formation of mono(hydroxyureato)iron(III) complex during the oxidation of HU with Fe(III) [40–42]. While for the majority of metal ions a high acidity destabilizes the hydroxamato complexes, the complexation of HU by V^{V} ions is favored by high acidity owing to the possible protonation of the coordinated oxo ligands. Our data can be plausibly explained by a rapid formation of two different hydroxamato complexes in equilibrium, i.e. $V^{V}O(OH)U^{+}$ and its protonated analog $V^{V}OU^{2+}$. The proton dissociation of $V^{V}OU^{2+}$ is characterized by a strong acidity of the coordinated water molecules, $pK_a = 0.632$. It seems worth to mention that the formation of a complex analogous to $V^{V}O(OH)U^{+}$ was

reported for thiourea [43], but the value of its stability constant was not reported.

Although complexes with the protonated form of *cis*-dioxovanadium ($OV^{V}OH$) were reported [44,45], and the protonation of $V^{V}O_2^+$ in strong acid was proposed on several occasions [37,46], no direct physical evidence to support the hypothesis has been provided so far. Begun and coworkers [47] have studied protonation of $V^{V}O_2^+$ in concentrated perchloric acid, but they proposed formation of a dimeric protonated V^{V} species, i.e. the $V_2O_3^{4+}$ ion. However, according to their results the protonated dimer starts to form at much higher acid concentrations than used in the present study, i.e at $[HClO_4] > 6$ M.

Here we present clear-cut evidence for the protonation of $V^{V}O_2^+$, based on a small but definite change in chemical shift of the ^{51}V-NMR spectrum for the ion observed upon acidification in aqueous perchloric acid medium. The observed difference in chemical shift is far too small to be related to the formation of $V_2O_3^{4+}$, for which the reported [39] chemical shift of -640 ppm lies far away from -545 ppm reported for the $V^{V}O_2^+$ ion [48].

For the first time the proton dissociation constant of the $V^{V}O(OH)^{2+}$ ion was determined ($pK_a = -0.15$), pointing to an even higher acidity of this species than of the $V^{V}OU^{2+}$ ion. In terms of the MO bonding scheme, the higher acidity of the $V^{V}O(OH)^{2+}$ ion could be accounted for by a higher basicity of the hydroxo than the oxo ligand, due to involvement of the oxo 2p orbitals in π-bonding that leaves only the non-bonding sp_σ hybrid for protonation. It should be noted that our pK_a value compares poorly with a value that can be calculated from the Sen Gupta and Chatterjee kinetic and thermodynamic data reported for oxidation of glyoxylic and pyruvic acids ($pK_a = +0.8$) [46]. However, their reported value seems to be too high; otherwise it certainly would not have escaped to be noticed in the numerous potentiometric titration studies performed so far.

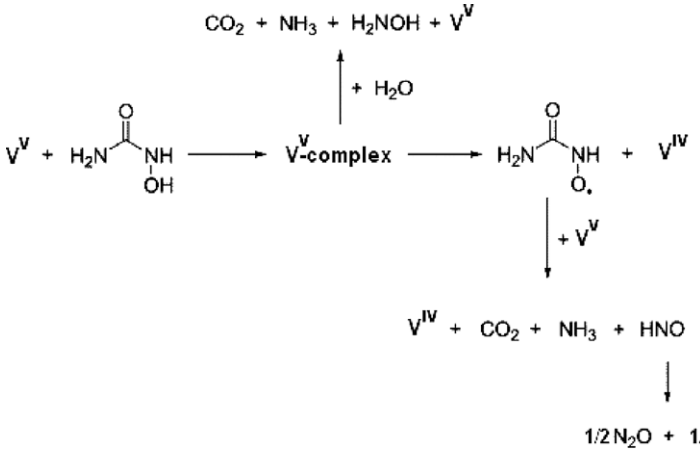
From the values of the proton dissociation constant for $V^{V}O(OH)^{2+}$ and K_1, the equilibrium constant $K = [VOU^{2+}][VO(OH)^{2+}]^{-1}[HU]^{-1}$ can be calculated to be 33 M^{-1}. A comparison of this value with $K_0 = 5$ M^{-1} indicates a slightly weaker affinity of HU for VO_2^+ than for $VO(OH)^{2+}$, possibly due to a strong *trans*-labilization exerted by the coordinated oxo ligand(s). In VO_2^+ at least one hydroxamato oxygen must be *trans* to the oxo ligand.

Our EPR measurements reveal that the reduction of V^{V} by HU proceeds via formation of a free radical wherein V^{V} can undergo only a one-electron reduction. The observation of a free radical eliminates a two-electron reduction mechanism in which the formed V^{III} would react with non-reacted V^{V} to give two V^{IV} species [49]. The EPR spectra also show that the free radical is immediately released from the complex.

We observed the formation of the same free radical in the redox reaction of HU with dioxovanadium(V) and hexacyanoferrate(III) ions in neutral aqueous solution [50]. The fact that the same free radical intermediate and reaction products are formed when HU is oxidized at drastically different acidities and with totally different types of oxidizing agents, points to the potential physiological relevance of our results by providing possible insight into an in vivo situation. Obviously, neither the nature of the oxidizing agent nor the acidity of the medium plays a critical role in the mechanism of the HU oxidation reaction.

A reaction scheme similar to the one already proposed for the oxidation of HU by hydrogen peroxide in neutral medium [8,51,52] and by Fe^{III} in acidic medium [40,41] could be invoked, but it should be noted that in the model oxidation of hydroxyurea by hydrogen peroxide, the formation of nitric oxide (NO) was observed. Focusing on the ligand, and for the sake of simplicity omitting all the protonation reactions, the complete coordination sphere of the vanadium ions, and the full stoichiometry of each step, the complete scheme for the reaction of HU with V^{V} can be depicted as in Scheme 2.

HNO is short-lived in aqueous solution due to the near diffusion-controlled rate of dimerization: $2HNO \rightarrow N_2O + H_2O$ [53]. A more recent paper reports a slower but still very fast reaction, viz. $k = 8 \times 10^6$ M^{-1} s^{-1} [54].



Scheme 2.

The lack of evidence for NO formation in our reaction and oxidation of HU by Fe(III), [41] contrary to the oxidation of HU with hydrogen peroxide, could be accounted for by a thermodynamic or kinetic rationale, i.e. either by a smaller standard reduction potential of V^V and Fe^{III} ions than peroxide (+1.00 V, +0.77 V, and +1.78 V, respectively) or by a much faster dimerization of HNO than its oxidation with V^V or Fe^{III} ions. For the NO, H^+/HNO couple in acidic aqueous solution, the former explanation would suggest a value for the standard reduction potential $E^0 \geqslant +1.0$ V. However, this value lies out of the range reported from as low as −1.6 V up to +0.4 V [55]. Interestingly, for a similar redox couple of an adduct of nitric oxide and nitroxyl, E^0(ONNOH, H^+/HONNOH) was reported to be +1.75 V [56]. Unless the reported values for the standard reduction potential are erroneous, our results would indicate that the formation of NO in the oxidation of HU depends mainly on kinetic factors, i.e. on the ratio between the rate of HNO oxidation and the rate of HNO dimerization. The observed formation of NO during oxidation of HU with H_2O_2 would therefore be a consequence of a fast reaction of HNO with H_2O_2 (owing to the high standard reduction potential of H_2O_2), whereas a lack of NO formation in the reaction of HU with Fe^{III} and VO_2^+ ions should be due to a slow oxidation of HNO by these two oxidants, because of their lower reduction potentials.

Our kinetic results (to be published elsewhere) indicate that the predominant pathway for oxidation of HU with VO_2^+ is an inner-sphere electron-transfer process, within the formed $V^VO(OH)U^+$ and V^VOU^{2+} complexes. A low stability of analogous complexes with HNO, i.e. $V^VO(OH)(NO)^+$ and $V^VO(NO)^{2+}$ may account for the slow oxidation of the formed HNO by V^V species, making the oxidation not competitive with the dimerization reaction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinorgbio.2006.05.008.

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