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Formation of free radicals during the oxidation of *N*-methylhydroxyurea with dioxovanadium(V) ions

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Abstract—Unlike the oxidation of hydroxyurea with dioxovanadium(V) ions, which proceeds through the formation of a single free radical, oxidation of *N*-methylhydroxyurea is characterized by consecutive formation of two oxygen-based free radicals, as recorded by EPR spectroscopy. Although the consumption of overall five V(V) ions per *N*-methylhydroxyurea molecule has been determined, the formation of NO, NO_2^- or NO_3^- was not observed. These results are in accordance with a previous suggestion that nitric oxide transfer requires an unsubstituted acylhydroxylamine group.

Hydroxyurea (HU, Fig. 1a), a hydroxamic acid inhibiting DNA synthesis, is widely used in cancer treatment.[1] It is also used to treat sickle-cell disease because it elevates fetal hemoglobin levels.[2] *N*-Methylhydroxyurea (NMHU, Fig. 1b) is a derivative of HU with approximately 50% the activity of HU.[3]

Although the mechanisms of the biological activities of hydroxyureas are still a matter of investigation, it is well known for these compounds to inhibit specifically human ribonucleotide reductase by reducing the tyrosyl free radical and the iron centre.[4,5]

The redox reaction of hydroxyurea with metal ions is characterized by the formation of an oxygen free radical, which is then transformed into the products. In the course of our investigation on the interactions of oxovanadium ions with hydroxamic acids,[6–8] we recently reported that the oxovanadium(V) ion is capable of oxidizing HU in an acidic medium. The reaction stoichiometry in the oxidation reaction was found to be 1:2 = HU:V^V,[9] whereas the results presented in this communication (Fig. 2) reveal that the reaction stoichiometry in the oxidation of NMHU with vanadium(V) ions is 1:5 = NMHU:V^V.

The stoichiometric ratio obtained was confirmed by EPR-spectral measurements. After completion of the redox reaction, the intensity of the well-known V(IV)-EPR lines compared to the signals of a reference

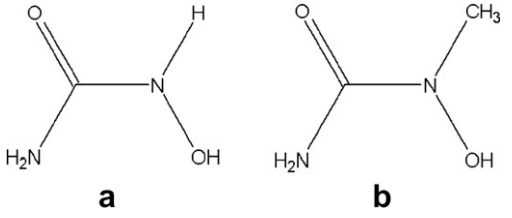

Figure 1. Hydroxyurea (a) and *N*-methylydroxyurea (b).

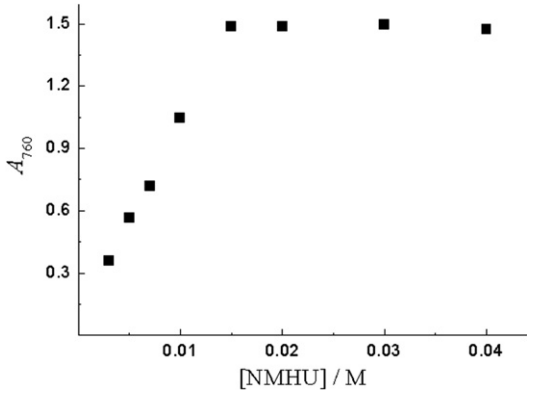

Figure 2. Absorbance corresponding to the concentration of $V^{IV}O^{2+}$ ions produced by oxidation of NMHU with V^V as a function of the NMHU concentration. Conditions: $[V^V] = 0.08$ M, $[H^+] = 1$ M, $\lambda = 760$ nm, $\theta = 25$ °C.

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$VO(ClO_4)_2$ solution revealed the formation of five V(IV) ions per oxidized HU molecule.

Although both hydroxamic acids exhibit similar pharmacological activities, their oxidations with V^V in acidic media are characterized, apart from the different reaction stoichiometries, by large differences in reactivity and in the reaction mechanisms. Here we present an investigation of the reaction mechanism of the oxidation of NMHU with dioxovanadium(V) and describe a peculiar behaviour of the observed intermediate-free radicals.

The reaction investigated has several distinct steps. As in the case of HU, during the first step proceeding on the stopped-flow time scale, a complexation reaction occurs characterized by the appearance of colour with the absorbance maximum being at 570 nm. The redox reaction starts in the second step by formation of an oxygen-based free radical (**A**) presented with a six resonance line EPR spectrum as shown in Figure 3.

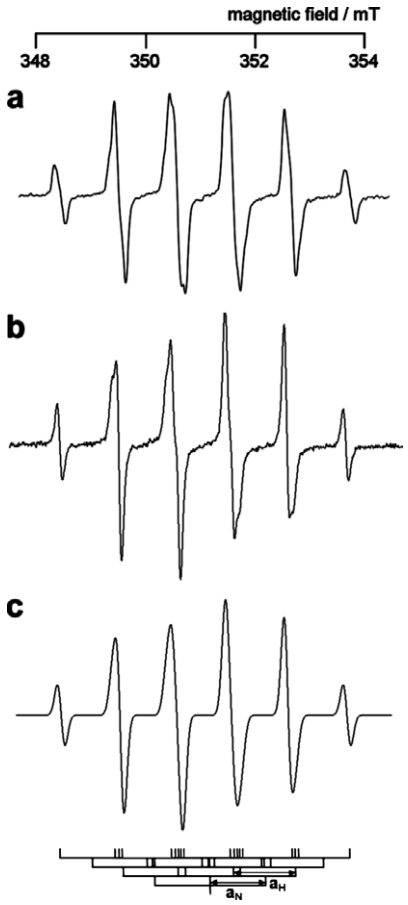


Figure 3. (a) EPR spectrum of oxygen radical **A** recorded at a microwave frequency of 9.86027 GHz, a microwave power of 10 mW and modulation amplitude of 0.1 mT. Conditions: $[V^V] = 0.002$ M, $[NMHU] = 0.001$ M, $[H^+] = 0.025$ M, $I = 2$ M. Bars beneath the spectrum indicate the positions of the six resonance lines formed by the interaction of the unpaired electron with the ^{14}N nucleus ($a_N = 0.98$ mT) and 3 equiv protons of the methyl group ($a_H = 1.08$ mT). (b) EPR spectrum taken in D_2O. (c) EPR spectrum simulated using the WinEPR SimFonia program using experimental hyperfine splittings and a line-width of 0.15 mT.

The six resonance line EPR spectrum of **A** is brought about by coupling of the unpaired electron with the ^{14}N nucleus ($a_N = 0.98$ mT), giving rise to the triplet structure of the resonance lines, further split into three doublets with equal hyperfine couplings ($a_H = 1.08$ mT). The equal doublet hyperfine couplings are characteristic for the interaction of the unpaired electron with three magnetically equivalent protons and these are assigned to the methyl protons of NMHU. No change in spectrum structure occurred on performing the measurements in D_2O, confirming the proposed assignment. The measured value of the *g*-factor of **A** (2.0068) is typical for oxygen-centred radicals indicating a significant amount of spin density located in an sp^3-like orbital of the hydroxylate-oxygen atom.[10] A similar oxygen-centred radical was observed in the reaction of HU with V^V.

Unlike the oxidation of HU where only one type of free radical was observed, oxidation of NMHU proceeds by formation of two different free radicals. To the best our knowledge, this is the first time that such a consecutive free-radical transformation has been observed in redox reactions of hydroxamic acids. The second paramagnetic species (radical **B**) is represented by the seven resonance line EPR spectrum (Fig. 4) with different hyperfine couplings evoked by a change of chemical structure in the vicinity of the unpaired electron.

The equal values of the observed *g*-factors for **A** and **B** are indicative of the same origin of the radical. However, the spectral patterns and the observed hyperfine splittings in **A** and **B** are rather different. Seven resonance lines in the EPR spectrum of **B**, with the intensity ratio 1:2:2:2:2:2:1, occur through coupling of the unpaired electron with ^{14}N ($a_N = 0.95$ mT) and 2 equiv protons

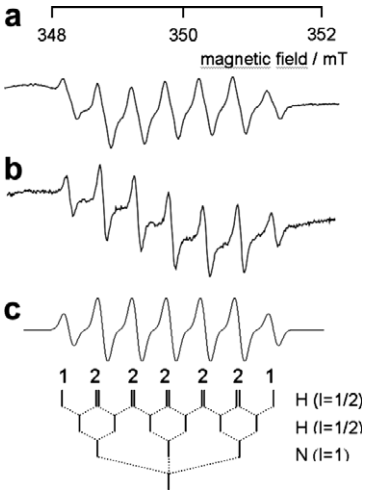


Figure 4. (a) EPR spectrum of oxygen radical **B** recorded at a microwave frequency of 9.82667 GHz, a microwave power of 10 mW and modulation amplitude of 0.1 mT. Conditions: $[V^V] = 0.01$ M, $[NMHU] = 0.001$ M, $[H^+] = 0.13$ M, $I = 2$ M. Bars beneath the spectrum represent the positions of seven resonance lines caused by coupling with the ^{14}N nucleus ($a_N = 0.95$ mT) and 2 equiv protons of the methylene group ($a_H = 0.47$ mT). (b) EPR spectrum taken in D_2O. (c) EPR spectrum simulated using the WinEPR SimFonia program using experimental hyperfine splittings and a line-width of 0.13 mT.

($a_H = 0.47$ mT). Almost the same nitrogen hyperfine value in two radicals indicates a similar spin density distribution, that is, sp^3 hybridization of nitrogen with the free electron pair located on that atom in **B** as well. Analogous with the oxidation of 5,5-dimethyl-1-pyrroline-*N*-oxide to its radical cation followed by addition of H_2O,[11] **B** can be formed by fast hydrolysis of a nitrone radical cation produced from **A** by single-electron transfer to V(V). Accordingly, the structure of **B** can best be depicted as $NH_2C(O)–N(O^{\cdot})–CH_2OH$.

However, the same EPR-spectral patterns shown in Figures 3 and 4 would also be observed if the unpaired spin was located mainly on the carbonyl oxygen. In such a case the observed hyperfine splitting would derive from 2 equiv amine H atoms. In order to resolve this ambiguity, the above experiments were repeated in D_2O. The deuterated reaction system showed the same EPR spectra, without extra splitting, but with somewhat sharper resonance lines, supporting the proposed assignation.

As clearly indicated in Figure 5, slow transformation of initially formed A into B can be followed easily. These results suggest that in the first step of the redox reaction radical **A** forms quickly, followed by slow transformation into **B**.

Under the experimental conditions of Figure 5 the formation of **A** is much faster than the transformation rate, but the latter is found to depend on the total concentrations of H^+, V^V and NMHU. Therefore, under some experimental conditions, the transformation reaction could be easily overlooked.

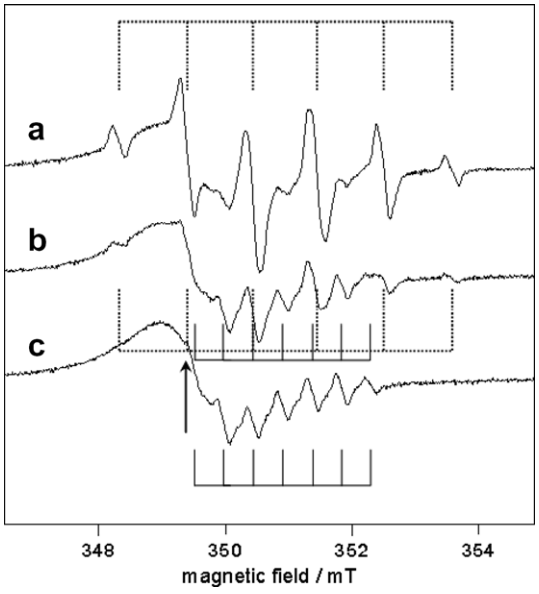


Figure 5. Transformation of radical **A** into radical **B** monitored by EPR. Spectra were recorded at a microwave frequency of 9.85465 GHz, a microwave power of 10 mW and modulation amplitude of 0.1 mT. Conditions: $[V^V] = 0.005$ M, $[NMHU] = 0.001$ M, $[H^+] = 0.025$ M, $I = 2$ M. EPR spectra were recorded after 45 s (spectrum a), 2 min (spectrum b) and 7 min (spectrum c) after mixing. The bars above or beneath the spectra represent the positions of the resonance lines of radicals **A** (dashed lines) and **B** (solid lines). The arrow indicates the position of the fourth resonance line of the well-known octet of the $V^{IV}O^{2+}$ ion.

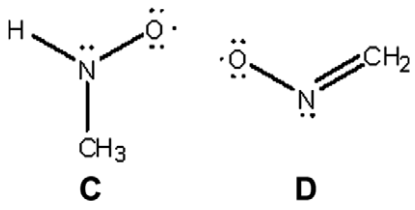


Figure 6. Structures of *N*-methylhydroxylamine (**C**) and formaldoxime (**D**) free radicals.

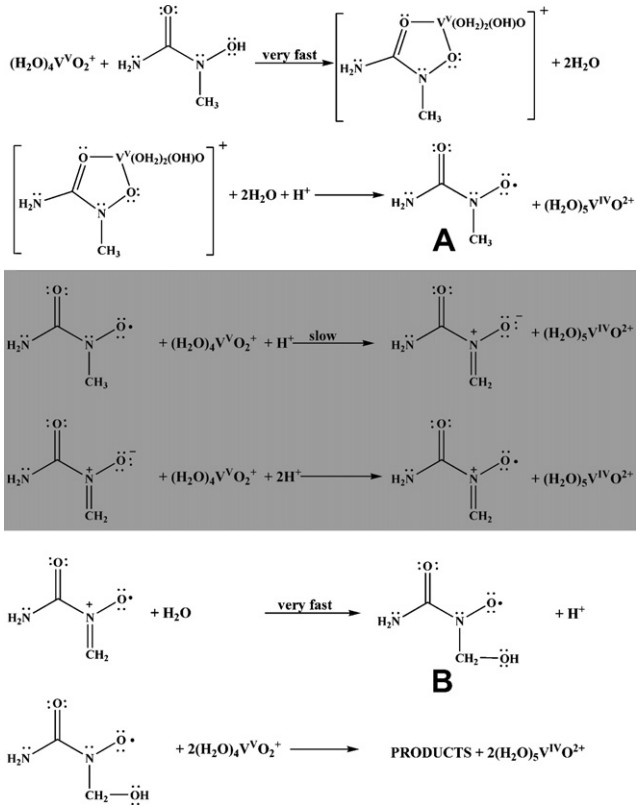
However, as the EPR spectra of **A** and **B** reflect only characteristics of the closest neighbourhood of the unpaired electron of the radicals' spins, the same effect of the solvent isotopic change could be observed if radical **A** hydrolyzes quickly to the *N*-methylhydroxylamine free radical (**C**) followed by its oxidation to formaldoxime free radical (**D**), Figure 6.

In order to clarify this point, several experiments have been undertaken. Under identical experimental conditions to those used for NMHU, neither the oxidation of *N*-methylhydroxylamine with vanadium(V) yielded **C**, nor did the oxidation of formaldoxime with vanadium(V) yield a free radical as characterized by the EPR spectrum shown in Figure 3. Nevertheless, vanadium(V) is capable of oxidizing both compounds as the emergence of the $V^{IV}O^{2+}$ characteristic blue colour was observed in the reaction solutions. Therefore, though the redox reactions take place, neither free radical **A** nor **B** is formed from these compounds. Furthermore, unlike in the oxidation of NMHU, formation of NO gas is observed during oxidation of formaldoxime with vanadium(V). This is in accordance with previously reported observations that NO exerting vasorelaxant effects in rat aorta is released from formaldoxime.[12]

Although during the overall oxidation of NMHU five electrons are transferred to vanadium(V) ions, the results indicate that neither nitric oxide gas, nitrites or nitrates were formed among the reaction products. This is in line with findings for the reactions of haemoglobin with NMHU, where NMHU or any radicals derived from it cannot transfer NO as the nitric oxide transfer requires an unsubstituted acylhydroxylamine group.[13]

Combining all of these results with the analogous results reported for HU[8,9] we propose that vanadium(V) oxidizes NMHU according to the reaction mechanism depicted in Scheme 1.

It should be noted that based on the presented results it cannot be distinguished whether the third and fourth steps (shaded area) shown in Scheme 1 involve two single electron-transfer steps yielding two VO^{2+} ions, or a $2e^-$ transfer followed by quick oxidation of V(III) with another V(V) ion, since under the experimental conditions the latter reaction is fast enough ($k = 10^4\ M^{-1}\ s^{-1}$)[14] not to interfere with the observed free radical transformation. However, since the EPR spectra



Scheme 1. Mechanism of the redox reaction between V^V and NMHU. The shaded area covers the tentative intermediates not confirmed by the measured EPR spectra.

of both free radicals eliminate their coordination to vanadium ions, and the outer-sphere $2e^-$ transfers are considered to be exceedingly slow reflecting a prohibitive Franck–Condon barrier, one may assume single electron transfers to be operative in radical transformation.

A much higher reactivity of the free radical derived from hydroxyurea than its analogue (radical **A**) derived from NMHU towards dioxovanadium(V) ions could possibly be related to the higher pharmacological activity of the former compound. A detailed kinetic study of the redox reaction is in progress and the results will be published elsewhere.

Acknowledgements

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