Free Rad. Res. Comms., Vol. 8, No. 2, pp. 123-131
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FREE RADICAL INTERMEDIATES IN THE OXIDATION OF N-METHYLHYDROXYLAMINE AND N,N-DIMETHYLHYDROXYLAMINE BY OXYHEMOGLOBIN

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(Received July 21, 1989; Accepted August 29, 1989)

Nitroxide radicals have been detected in the methemoglobin formation reaction between oxyhemoglobin and the substituted hydroxylamine compounds, N-methylhydroxylamine and N,N-dimethylhydroxylamine, by ESR spectroscopy. The stability of these nitroxide radicals was considerably higher than that of the $NH_2O^{\cdot}$ radical derived from unsubstituted hydroxylamine. Only in the case of N-methylhydroxylamine the detection of the nitroxide radical required the use of a flow system, because the radical was found to undergo a rapid degradation with the concomitant formation of a secondary product, the β-aminonitroxide $CH_3NO^{\cdot}CH_2NH_2$. The nitroxide radical derived from N,N-dimethylhydroxylamine and oxyhemoglobin was stable for more than 1 hour. In addition, formation of low-spin iron-(III)-complexes from methemoglobin and excess substituted hydroxylamine was observed in both cases. Neither N-methylhydroxylamine nor N,N-dimethyldroxylamine formed the hemoglobin-nitric oxide complex found with unsubstituted hydroxylamine. Parallels and differences in the reaction path of un-, mono- and disubstituted hydroxylamines are discussed.

KEY WORDS: ESR, N-methylhydroxylamine, N,N-dimethylhydroxylamine, free radicals, methemoglobin formation.

INTRODUCTION

In a recent paper[1] we described the paramagnetic intermediates formed in the reaction between hydroxylamine and oxyhemoglobin, namely the dihydronitroxide free radical ($NH_2O^{\cdot}$), the methemoglobin-hydroxylamine adduct (MetHb-NH_2OH), the hemoglobin-nitric oxide complex, and the $Fe(NO)_2X_2$ (X = inorganic anions) complex. We extended our investigations to the mono- and disubstituted compounds N-methylhydroxylamine and N,N-dimethylhydroxylamine in order to further corroborate the reaction scheme elaborated for hydroxylamine and oxyhemoglobin. We expected two major influences of the aliphatic substituents of mono- and dimethylhydroxylamine. First, the stabilizing effect[2] on the nitroxide radical ($RNHO^{\cdot}$ or $R_2NO^{\cdot}$) should facilitate the detection of this radical so as to allow measurements in a stationary system or at lower hydroxylamine concentrations. Second, differences in the following steps of the reaction can be expected, mainly the decay of the nitroxide radical and the oxidation of the methemoglobin with substituted hydroxylamines, which is not likely to be transformed into the nitric oxide adduct ($Hb^{2+}NO$)

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as we have reported for the unsubstituted hydroxylamine complex.[1] Anderson and Norman[3] reported the formation of $CH_3NO^{\cdot}CH_2NH_2$ free radicals as secondary products from the nitroxide radical and excess N-methylhydroxylamine in a system containing N-methylhydroxylamine and titanium(III) ions. We wanted to know whether this secondary radical is also formed in the oxyhemoglobin system and if N,N-dimethylhydroxylamine can also form this type of dimer.

MATERIALS AND METHODS

N-methylhydroxylamine and N,N-dimethylhydroxylamine were obtained from Aldrich Chemical Co. Bovine hemoglobin was prepared in a modified procedure described by Eyer *et al.*[4] for human hemoglobin. Bovine red cells were washed five times with twice the amount of 0.2 M phosphate buffer, pH 7.4. The cells were sonicated in distilled water and 10 g of Celite were added to 250 ml of the hemolysate. The mixture was stirred for 20 min and then centrifuged for 30 min at 15,000 g. Purified hemoglobin was prepared by chromatography of the hemolysate on $DEAE_{52}$-cellulose. 10 ml of the hemolysate were applied to a column (26 mm I.D.) containing 50 g of $DEAE_{52}$ cellulose (Serva) preequilibrated with 10 mM Tris/HCl pH 8.3 and eluted with 0.1 M Tris/HCl pH 7. The fractions were tested for catalase and SOD activity[5] and only those with a catalase activity $K < 1$ and no detectable SOD activity were pooled. For the experiments where phosphate buffer was used, the pooled fractions were chromatographed on Sephadex G-25 and eluted with 0.2 M phosphate buffer, pH 7.4. Oxyhemoglobin was determined at 540 nm, the methemoglobin content by the increase in absorbance at 540 nm caused by the addition of cyanide.[6] The ESR experiments were carried out in a Bruker ER 200 D-SRC 9/2.7 spectrometer operating at 9.6 GHz with 100 kHz modulation frequency equipped with a rectangular TE_{102} microwave cavity. For the measurements of the g-values at room temperature, 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) was used as an internal standard ($g = 2.0055$).[7] For the liquid nitrogen temperature measurements, 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used ($g = 2.0036$).[8] For the flow experiments a quartz mixing flat-cell was chosen and for the liquid nitrogen temperature measurements a finger dewar equipped with a quartz test tube. Computer simulations of ESR spectra and multi-scan experiments were carried out with the Bruker data system ESR 1600.

RESULTS

Measurements at Room Temperature

Figure 1A shows the ESR spectrum obtained when N-methylhydroxylamine (0.9 M, pH 7.4, containing 1 mM DETAPAC) and oxyhemoglobin (4.74 mM in 0.2 M phosphate buffer pH 7.4, containing 1 mM DETAPAC) were mixed in a quartz mixing cell at a flow rate of approximately 6 ml/min. The g-value of this radical is $g = 2.0055$ and the splitting constants are $a_N = a_H = 14.8$ G. The same values have been reported for the $CH_3NHO^{\cdot}$ radical,[9] obtained in aqueous solution containing potassium hexacyanoferrate-(III) and N-methylhydroxylamine.

When the flow was stopped, the ESR spectrum disappeared gradually within a few

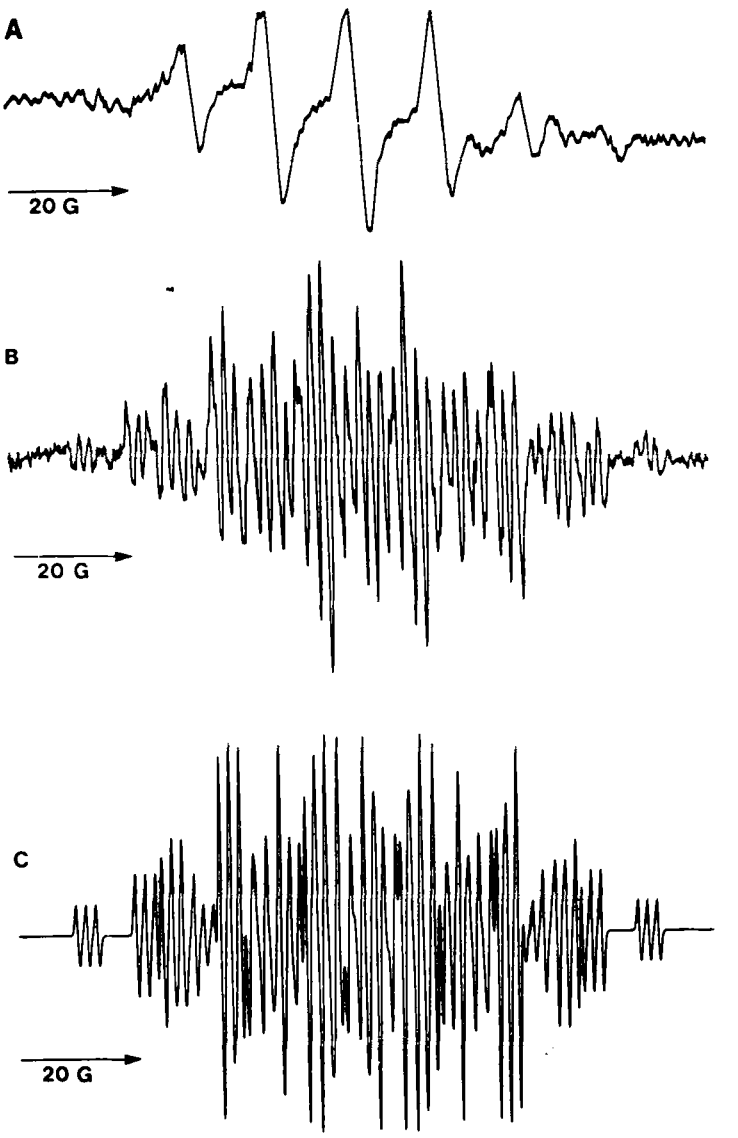

FIGURE 1 N-methylhydroxylamine-derived radicals. A): ESR-spectrum of the N-methylhydronitroxide radical. The flow rate was 6 ml/min total. The two components were: 1) oxyhemoglobin solution (4.74 mM, pH 7.4, containing 1 mM DETAPAC) and 2) N-methylhydroxylamine (0.9 M, pH 7.4, containing 1 mM DETAPAC). The spectrometer settings were: scan range, 200 G, modulation amplitude, 4 G; receiver gain, 1.25×10^6; microwave power, 20 mW; time constant, 1 sec; scan rate, 24 G/min. B): ESR-spectrum of the N-methyl-N-β-aminomethylnitroxide radical. The incubation mixture was the same as in A), except that the flow was stopped. The spectrometer settings were: scan range, 120 G; modulation amplitude, 0.5 G; receiver gain, 3.2×10^6; microwave power, 20 mW; time constant, 2 sec; scan rate, 7.2 G/min. C): Computer simulation of the ESR spectrum shown in B). The parameters used were: $a_N = 16.6$ G; $a_H(3) = 14.4$ G; $a_H(2) = 10.0$ G; $a_N = 1.7$ G; linewidth $\Delta H = 0.9$ G; 100% Gaussian line shape.

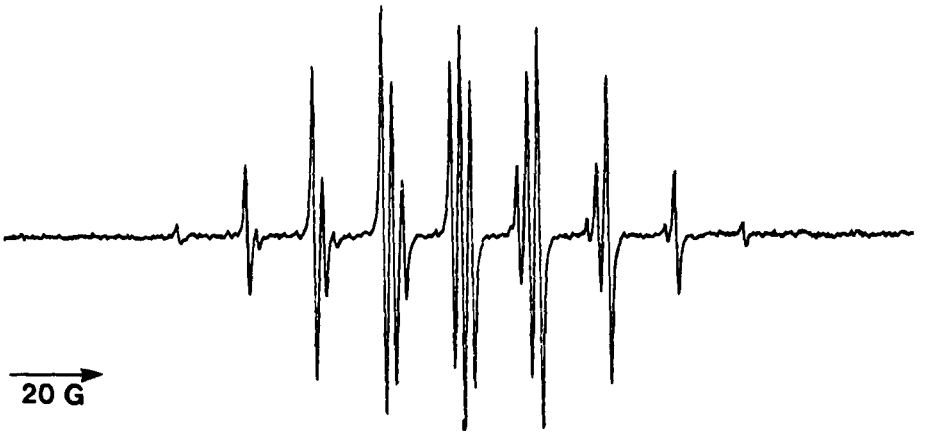

FIGURE 2 ESR spectrum of the N,N-dimethylnitroxide radical. Stationary system: the incubation mixture contained N,N-dimethylhydroxylamine (20 mM), and oxyhemoglobin (2 mM), in 0.2 M phosphate buffer, pH 7.4, containing 1 mM DETAPAC. The spectrometer settings were: scan range, 200 G; modulation amplitude, 0.25 G; receiver gain, 1×10^5; microwave power, 20 mW; time constant, 0.16 sec; scan rate, 72 G/min.

minutes and a secondary radical was obtained. The stability of this secondary radical was much higher than that of the primary radical and its spectrum is shown in Fig. 1B. In order to interpret this very complex ESR spectrum, we repeated the experiment in D_2O buffer (pD = 7.4). The spectrum did not change except for a slightly lower linewidth (spectrum not shown). This means that the coupling constants of the exchangeable protons are smaller than the spectral resolution. The interpretation of the spectrum was done using computer simulation shown in Fig. 1C. The parameters used to obtain the best fit for the spectrum shown in Fig. 1B were: a_N = 16.6 G; $a_H(3)$ = 14.4 G; $a_H(2)$ = 10.0 G; and a_N = 1.7 G. The line-width chosen was ΔH = 0.9 G assuming 100% Gaussian line shape. The observed g-value was g = 2.0055. Based on the shape of the two ESR spectra in 1B and 1C together with the paramagnetic characteristics evaluated by computer simulation we concluded that the structure of this secondary radical is that of a β-aminonitroxide ($CH_3NO^{\cdot}CH_2NH_2$). This radical has been described by Waters[9] and by Poupko *et al.*,[10] who reported similar spectral parameters. Poupko *et al.* reported an additional splitting of the amino protons $a_H(2)$ = 0.45 G. This is in agreement with the line-width narrowing that we observed in D_2O which can be explained by unresolved splittings of the exchangeable amino protons ($a_H(2) < 0.5$ G). The formation of this radical most probably stems from self trapping with excess hydroxylamine since we could not observe this species when we used lower concentrations of N-methylhydroxylamine.

Fig. 2 shows the ESR spectrum obtained in a stationary system containing 20 mM N,N-dimethylhydroxylamine and 2 mM oxyhemoglobin in 0.2 M phosphate buffer, pH 7.4, containing 1 mM DETAPAC. The radical is remarkably stable and no secondary species was detected. The g-value was 2.0054 and the coupling constants were: a_N = 17.0 G; and $a_H(6)$ = 14.8 G. The corresponding structure of this radical is $(CH_3)_2NO^{\cdot}$ and its increased stability compared to the un- and monosubstituted

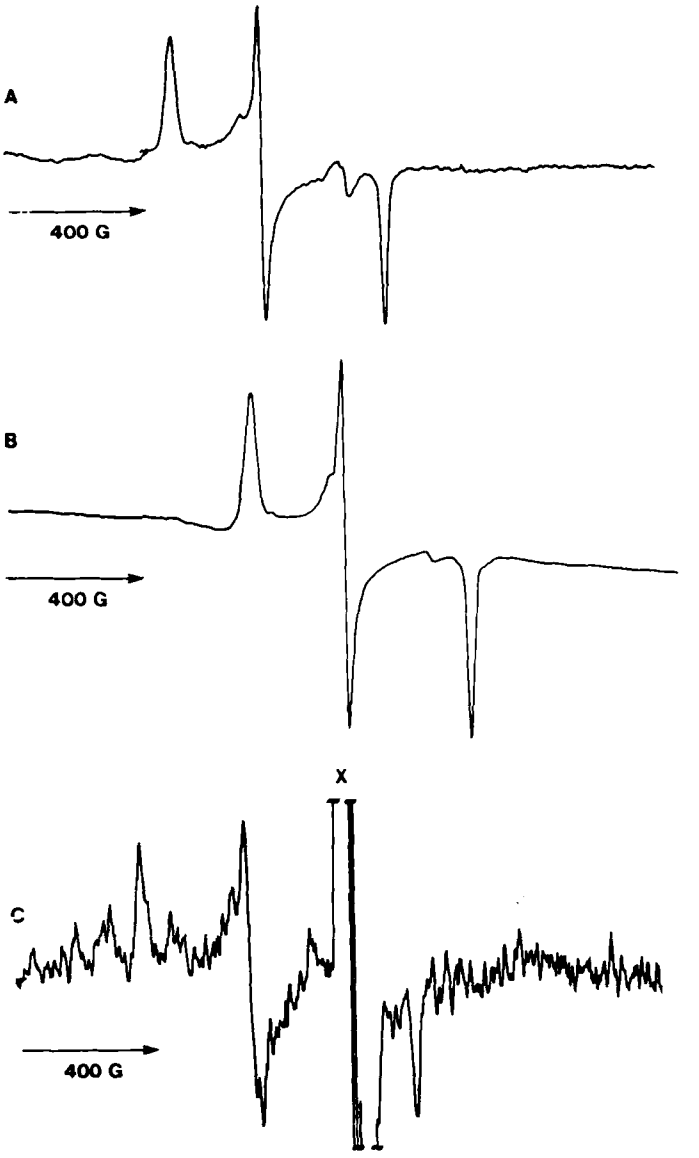

FIGURE 3 ESR spectra of the low-spin ferric iron complexes. A): The methemoglobin-N-methylhydroxylamine complex. The incubation mixture contained oxyhemoglobin (2 mM) and N-methylhydroxylamine (0.2 M). The spectrum was recorded at 77° K. The spectrometer settings were: scan range, 2000 G; modulation amplitude, 5 G; receiver gain, 6.3×10^4; microwave power, 20 mW; time constant, 0.64 sec; scan rate 357 G/min. B): The methemoglobin-N-methylhydroxylamine complex. The incubation mixture contained methemoglobin (1.77 mM) and N-methylhydroxylamine (0.33 M). The spectrum was recorded at 77° K. The spectrometer settings were: scan range, 2000 G; modulation amplitude, 2.5 G; receiver gain, 1.6×10^5; microwave power, 10 mW; time constant, 1.0 sec; scan rate, 240 G/min. C): The methemoglobin-N,N-dimethylhydroxylamine complex. The incubation mixture contained oxyhemoglobin (2 mM) and N,N-dimethylhydroxylamine (0.2 M). The spectrum was recorded at 77° K. The spectrometer settings were: scan range, 2000 G; modulation amplitude, 5 G; receiver gain, 3.2×10^5; microwave power, 20 mW; time constant 1.31 sec; scan rate, 357 G/min.

nitroxide radicals can be explained by the electron-donating effect (+I-effect) of the methyl groups[11] as well as some steric hindrance that decreases the reactivity.

Measurements at Liquid Nitrogen Temperature

The ESR spectrum shown in Figure 3A was obtained when a solution containing N-methylhydroxylamine (0.2 M, pH 7.4) and oxyhemoglobin (2 mM) was incubated at pH 7.4 for 45 sec. and then frozen in liquid nitrogen. The spectrum was recorded at 77° K and exhibited three distinct g-values: $g_1 = 2.38$, $g_2 = 2.17$, and $g_3 = 1.93$, typical of low-spin ferric iron complexes. In our previous paper[1] we assigned the analogous complex formed from hydroxylamine and oxyhemoglobin to the

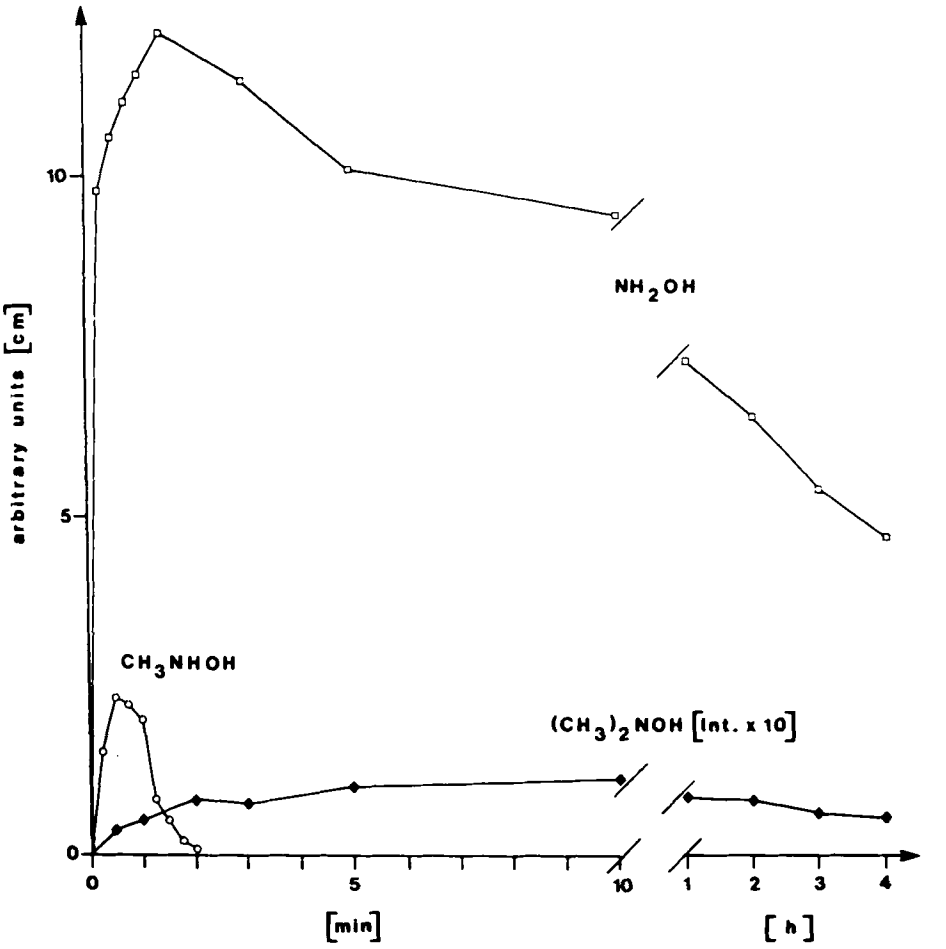

FIGURE 4 Time course of the reaction between oxyhemoglobin (2 mM) and hydroxylamine (□), N-methylhydroxylamine (○), and N,N-dimethylhydroxylamine (◆). Concentrations were 0.2 M, pH was 7.4. The reaction temperature was 25° C. The samples were frozen at different intervals and their ESR spectra were recorded at 77° K. The intensities represent the height of the most intense peak in arbitrary units.

methemoglobin-hydroxylamine adduct (g-values 2.46, 2.20 and 1.91). Figure 3B shows the formation of the same complex as in Figure 3A from methemoglobin (1.77 mM) and N-methylhydroxylamine (0.33 M). The corresponding complex of N,N-dimethylhydroxylamine with methemoglobin is shown in Figure 3C. In this case, the complex is formed only as a minor product, the main reaction product being the dimethylnitroxide radical (center line marked "x", off scale). The incubation mixture contained oxyhemoglobin (2.0 mM) and N,N-dimethylhydroxylamine (0.2 M, pH 7.4) and was frozen after 4 min. The observed g-values were: $g_1 = 2.49$, $g_2 = 2.22$, and $g_3 = 1.89$. After approximately 10 min incubation, a second low-spin complex became visible, with the g-values $g_1 = 2.41$, $g_2 = 2.20$, and $g_3 = 1.92$, most probably a degradation product of the first species (spectrum not shown).

In Figure 4 the time course of the methemoglobin complexes with the substituted hydroxylamines is depicted. In order to be able to compare the relative reactivities, the initial concentrations of the hydroxylamines (0.2 M) and oxyhemoglobin (2.0 mM) were equal in all cases. As can be clearly seen, the highest concentration of this low-spin ferric iron complex was obtained with unsubstituted hydroxylamine. The maximum was reached after about 90 sec, followed by a slow decrease in the range of some hours. With N-methylhydroxylamine the maximum was reached after only 30 sec, and after 2 min the concentration was already below the detection level. With N,N-dimethylhydroxylamine only minor concentrations were obtained with the maximum reached after about 10 min. The following slow decrease was accompanied by the formation of a variety of paramagnetic secondary products.

DISCUSSION

With substituted hydroxylamine (N-methylhydroxylamine and N,N-dimethylhydroxylamine) and oxyhemoglobin the same type of radical was formed as with unsubstituted hydroxylamine: the nitroxide radical $R_1R_2NO^{\cdot}$. The stability of this nitroxide radical increased from hydroxylamine (seconds or less) and N-methylhydroxylamine (less than one minute) to N,N-dimethylhydroxylamine (stable for hours). In agreement with our previous results with unsubstituted hydroxylamine[1] we assume the formation of the nitroxide radical $R_1R_2NO^{\cdot}$ being the first step of the reaction sequence, because the ESR spectrum of this radical can be observed immediately after the reaction has been started:

$$HbO_2 + R_1R_2NOH + H^+ \rightarrow R_1R_2NO^{\cdot} + MetHb + H_2O_2$$

As a possible non-paramagnetic reaction intermediate we assume the formation of hydrogen peroxide, although we were not able to detect it with conventional methods. Catalase, for example, cannot be used because it forms catalytically inactive complexes with hydroxylamine.[12] Second, an accumulation of free hydrogen peroxide to spectroscopically detectable levels is very unlikely, because excess hydroxylamine compound will rapidly be oxidized via the highly reactive methemoglobin-hydrogen peroxide adduct reported by Shiga *et al.*[13] Although this complex is paramagnetic ($g = 2.00$), its ESR spectrum cannot easily be detected due to strong interference with the nitroxide radicals that absorb in the same region.

Compared to the unsubstituted nitroxide, the degradation of the substituted nitroxide radicals must take a different route, since the formation of nitrogen gas is no longer possible without the rearrangement of the whole molecule. In the case of

N-methylhydroxylamine a possible route to stable products is the disproportionation to formaldoxime and N-methylhydroxylamine:

$$2CH_3NHO^{\cdot} \rightarrow CH_2{=}N{-}OH + CH_3NHOH$$

Further investigations using additional analytical techniques are required to determine the non-paramagnetic reaction intermediates.

The reaction leading to the formation of the secondary product is not fully understood. One possible reaction sequence could be the disproportionation of excess N-methylhydroxylamine in the absence of competing dioxygen and subsequent condensation of the so-formed products followed by oxidation to the β-aminonitroxide radical. An explanation for the increased stability of the N,N-dimethylhydroxylamine-derived nitroxide radical is the fact that the above-mentioned reaction sequence is not possible with disubstituted compounds.

A further step in the overall-reaction described in this paper is the formation of a low-spin ferric iron complex from methemoglobin and excess of either unsubstituted or substituted hydroxylamine:

$$MetHb + R_1R_2NOH \rightarrow MetHb - R_1R_2NOH$$

The ESR spectra of this complex were observed with all the investigated compounds and it was also shown that the degradation of this complex proceeded with different velocity. The respective degradation products could not be identified, but it was shown that the nitric oxide adduct ($Hb^{2+}NO$) observed in the reaction of oxyhemoglobin with unsubstituted hydroxylamine was formed neither with N-methylhydroxylamine nor with N,N-dimethylhydroxylamine. A second low-spin ferric iron complex was formed with N,N-dimethylhydroxylamine and oxyhemoglobin, the concentration of which increased concomitantly with the disappearance of the primary product. This permits the assumption that the secondary complex is a degradation product of the primary adduct. We were not able to identify this secondary species, but its spectral parameters clearly exclude the monomethyl- and unsubstituted hydroxylamine complexes.

In summary, the reaction scheme established with unsubstituted hydroxylamine in our previous paper[1] was confirmed by the results obtained with the substituted compounds. In all cases, where the methyl substituents put no obstacle to the reaction, the substituted hydroxylamines behaved similarly, namely in the formation of the nitroxide radical and in the reaction with methemoglobin to the low-spin iron-(III) complex. The oxidation of this complex to the $Hb^{2+}NO$ adduct, on the other hand, where the substituents would have to be eliminated, is not observed with the substituted hydroxylamines.

Acknowledgements

The authors wish to thank Prof. Dr. Manfred Gemeiner and Ing. Ingrid Miller for expert technical assistance in the chromatography procedures. This research was supported by the Oesterreichische Fonds zur Förderung der wissenschaftlichen Forschung; Projekt Number: P07150-ME.

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Accepted by Prof. H. Sies