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DETECTION OF FREE RADICALS AS INTERMEDIATES IN THE METHEMOGLOBIN FORMATION FROM OXYHEMOGLOBIN INDUCED BY HYDROXYLAMINE

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Abstract—Four distinct paramagnetic intermediates could be observed in the reaction between oxyhemoglobin and hydroxylamine using ESR spectroscopy. The radical species exhibited different stability properties thus different techniques were required for their detection. Two of them were identified as the hydronitroxide radical ($NH_2O^{\cdot}$) and the hemoglobin–nitric oxide complex (Hb^{2+}–NO). The third one is a low-spin iron-(III)-complex, possibly the methemoglobin–hydroxylamine adduct. A fourth paramagnetic species was detected only in the absence of the iron chelator DETAPAC thus indicating that free iron ions were responsible for the formation of this intermediate. The same species was observed when a Fenton system was used to generate the radicals. This species was identified as being the $Fe(NO)_2X_2$ complex described in the literature (X = inorganic anions such as OH^- or PO_4^{3-}). The identification of the radical intermediates detected in the hydroxylamine-induced methemoglobin formation contributes to a more detailed understanding of the reaction sequence.

The reactions between hydroxylamine and the different forms of hemoglobin have been investigated by several authors [1–3]. Bazylinski *et al.* [1] studied the reaction of hydroxylamine with deoxyhemoglobin and methemoglobin under anaerobic conditions and observed a two-step reaction in which hemoglobin cycles between deoxyhemoglobin and methemoglobin and hydroxylamine is converted into ammonia, nitrogen gas and small amounts of nitrous oxide. No reaction mechanism has yet been reported for the reaction between oxyhemoglobin and hydroxylamine, but nitrogen gas is expected to be the main reaction product due to its high thermodynamic stability. It is assumed that free radical intermediates are involved in this reaction since the heme iron is a one-electron reactant and two electrons are required to form nitrogen gas. The present study uses electron spin resonance spectroscopy as a tool to detect these paramagnetic species. In combination with a rapid flow technique or using liquid nitrogen temperature it is possible to investigate short-lived species that cannot be detected at room temperature in the stationary system. Four different radical species have been found and are discussed in the present paper.

MATERIALS AND METHODS

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. Ten ml of the hemolysate were applied to a column (26 mm i.d.) containing 50 g of $DEAE_{52}$ cellulose (Serva, Heidelberg, F.R.G.) 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 as in [5] and [6] 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 [7]. 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). For the liquid nitrogen temperature measurements, 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used (g = 2.0036). For the flow experiments a quartz mixing flat-cell was chosen and for the temperature measurements either a finger dewar equipped with a quartz test tube (at 77°K) or the Bruker variable temperature unit operating in the range between 110°K and room temperature.

RESULTS

Measurements at room temperature

The flow experiments at room temperature were carried out at a flow rate of approximately 20 ml/min total. Figure 1(A) shows the ESR spectrum of

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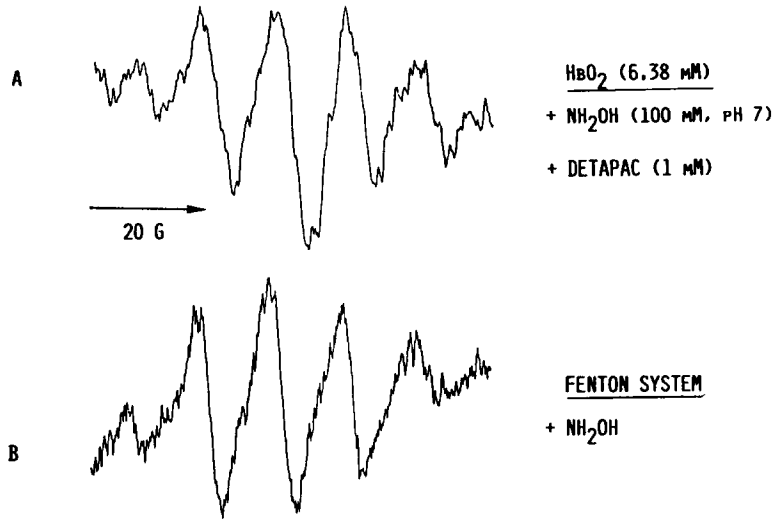

Fig. 1. ESR spectrum of the $NH_2O^{\cdot}$ radical. (A) The flow rate was 20 ml/min total. The two components were: (1) oxyhemoglobin solution (6.32 mM, containing 1 mM DETAPAC) and (2) hydroxylamine (100 mM, pH 7, containing 1 mM DETAPAC). The spectrometer settings were: scan range, 100 G; modulation amplitude, 5 G; receiver gain, 5×10^6; microwave power, 20 mW; time constant, 1 sec; scan rate, 30 G/min. (B) Fenton System: The flow rate was 20 ml/min total. The two components were: (1) hydrogen peroxide (10 mM) and (2) iron-(II)-sulfate (10 mM) and hydroxylamine (100 mM). The final pH was 2.7. The spectrometer settings were: scan range, 100 G; modulation amplitude, 2.5 G; receiver gain, 4×10^6; microwave power, 20 mW; time constant, 1 sec; scan rate, 12 G/min.

a transient radical obtained when hydroxylamine (0.1 M, pH 7, containing 1 mM DETAPAC) and oxyhemoglobin (6.38 mM, containing 1 mM DETAPAC) were mixed in a quartz mixing cell. The signal disappeared immediately when the flow was stopped, indicating a high reactivity of this species. The g-value of this radical is g = 2.0062 and the splitting constants are $a_N = a_H = 12.6$ G, roughly the same as obtained by Gutch and Waters [8] as well as by Adams *et al.* [9], who reported $a_N = a_H = 11.9$ G for the hydronitroxide radical ($NH_2O^{\cdot}$) in methanol. In that case, ammonium hexanitratocerate (IV) was used to oxidize hydroxylamine to the $NH_2O^{\cdot}$ radical. They also used a flow apparatus and even higher flow rates of *ca.* 200 ml/min. In order to identify the spectrum in Fig. 1(A) we produced the $NH_2O^{\cdot}$ radical using a Fenton system. The results are shown in Fig. 1(B), where hydroxylamine (100 mM), iron-(II)-sulfate (10 mM) and hydrogen peroxide (10 mM) were mixed in the flow cell and the spectrum recorded at room temperature. The spectrum obtained was nearly identical and also exhibited the same splitting constants as in Fig. 1(A). The fact that an identical ESR spectrum was obtained can be regarded as good evidence of the identity of the $NH_2O^{\cdot}$ radical ($a_N = a_H = 12.6$ G).

Figure 2(A) shows the ESR spectrum obtained when a higher scan range (400 G) was chosen under otherwise similar conditions as in Fig. 1(A). In addition to the lines stemming from the $NH_2O^{\cdot}$ radical (marked "×"), a strong single line (marked "○") with the g-value, g = 2.032, was obtained. This value is characteristic for the $Fe(NO)_2X_2$ complexes described by McDonald *et al.* [10], where X represents variable anions such as phosphate, sulfate, chloride or hydroxide (at higher pH). In order to investigate the role of free iron ions in this reaction sequence we designed two additional experiments. In Fig. 2(B), a Fenton system consisting of hydroxylamine (2 M, pH 6), hydrogen peroxide (10 mM), and iron-(II)-chloride (10 mM) was used to generate the two different radicals shown above. Here, the strong absorption line of the $Fe(NO)_2X_2$ complex proves undoubtedly that free irons are responsible for its formation. In the experiment shown in Fig. 2(C), 1 mM DETAPAC was added to both the hydroxylamine (100 mM, pH 7) and the oxyhemoglobin solution (6.32 mM) in order to chelate free iron ions. No $Fe(NO)_2X_2$ complex was formed but the five absorption lines of the $NH_2O^{\cdot}$ radical are still visible. This proves that oxyhemoglobin forms only the $NH_2O^{\cdot}$ species (marked "×"), but free iron ions are required to form the $Fe(NO)_2X_2$ complex (marked "○").

Measurements at liquid nitrogen temperature

Figure 3(A) shows the ESR spectrum obtained when a solution of oxyhemoglobin (6.32 mM) was allowed to react with hydroxylamine (100 mM, pH 7) for 2 min. The reaction was stopped by rapid freezing and the ESR spectrum was recorded at 110°K. The species observed is the hemoglobin-nitric oxide adduct described by Kon [11].

Figure 3(B) shows the ESR spectrum of the hemoglobin–nitric oxide adduct independently synthesized by mixing a solution of deoxyhemoglobin (8.9 mM) with nitric oxide gas in an oxygen free atmosphere. The spectrum was recorded at 113°K. It is identical to the spectrum shown in Fig. 3(A) again with a shoulder at $g_1 = 2.060$ and the minimum at $g_2 = 1.986$. When increasing the scan range to 1500 G additional ESR absorption lines could be

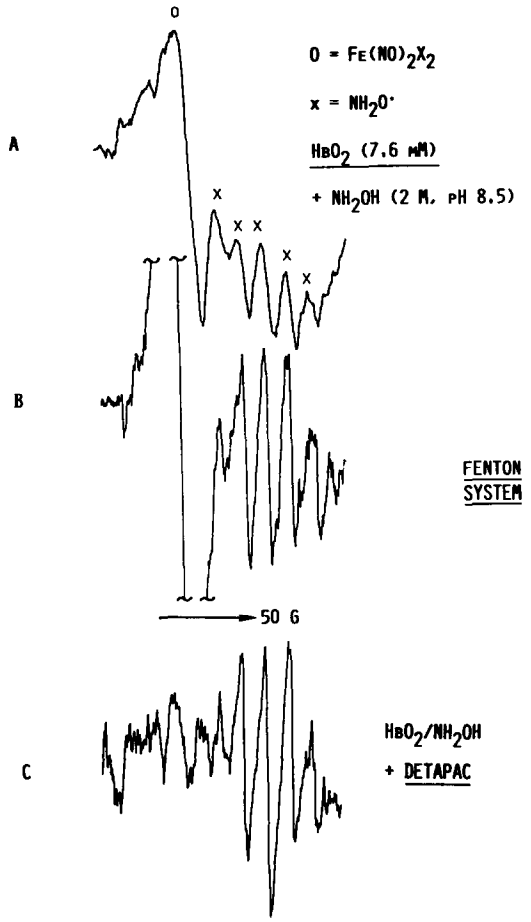

Fig. 2. ESR spectrum of the $NH_2O^{\cdot}$ radical and the $Fe(NO)_2X_2$ species. (A) The flow rate was 20 ml/min total. The two components were: (1) oxyhemoglobin (7.6 mM) and (2) hydroxylamine (2 M, pH 8.5). The spectrometer settings were: scan range, 400 G; modulation amplitude, 5 G; receiver gain, 1.25×10^6; microwave power, 20 mW; time constant, 1 sec; scan rate, 48 G/min. (B) The flow rate was 20 ml/min total. The two components were: (1) hydroxylamine (2 M, pH 6) and (2) hydrogen peroxide (10 mM) and iron-(II)-chloride (10 mM). The spectrometer settings were: scan range, 400 G; modulation amplitude, 2 G; receiver gain, 5×10^6; microwave power, 20 mW; time constant, 1 sec; scan rate, 48 G/min. (C) The flow rate was 20 ml/min total. The two components were: (1) oxyhemoglobin (6.32 mM, containing 1 mM DETAPAC) and (2) hydroxylamine (100 mM, pH 7, containing 1 mM DETAPAC). The spectrometer settings were: scan range, 40 G; modulation amplitude, 5 G; receiver gain, 5×10^6; microwave power, 20 mW; time constant, 1 sec; scan rate, 48 G/min.

detected. The respective spectrum shown in Fig. 3(C) exhibits besides the hemoglobin–nitric oxide adduct of Fig. 3(A,B) (marked "○") three additional lines (marked "×"). They are derived from a low-spin ferric heme species that we tentatively assign to a methemoglobin–hydroxylamine complex, since the same ESR spectrum was obtained when a solution of methemoglobin (5 mM) was mixed with hydroxylamine (100 mM, pH 7). The three g-values are $g_1 = 2.46$, $g_2 = 2.20$ and $g_3 = 1.91$. The time course of the ESR-intensity of these two species can be seen in Fig. 4, where the line marked "▼" represents the peak-height of the Hb–NO complex, whereas the line marked "○" stems from the middle peak of the MetHb–NH_2OH adduct. It is evident that the decrease of the latter is accompanied by a concomitant increase of the former, suggesting that the Hb–NO species results from the decomposition of the MetHb–NH_2OH adduct.

DISCUSSION

The presented results show the formation of four distinct paramagnetic species in the reaction of oxyhemoglobin with hydroxylamine. The first step in the reaction sequence seems to be the formation of the hydronitroxide radical ($NH_2O^{\cdot}$). Its formation rate must be relatively high since it can successfully compete with its rapid decomposition, this fact being a prerequisite for the successful use of the rapid flow technique. The second step is the rapid disappearance of the $NH_2O^{\cdot}$ radicals. The main pathway is possibly the formation of nitrogen gas and water as it is the case when no other reactants are present [12], but additional reactions with reactive groups at the hemoglobin moiety might also occur. The formation of the hemoglobin–nitric oxide complex on the other hand is a much slower process. This can be concluded from the fact that its ESR spectrum cannot be observed when the rapid flow technique is used. When the flow is stopped, the intensity of its ESR spectrum increases only gradually, thus indicating that this complex cannot be the direct product of the hydronitroxide ($NH_2O^{\cdot}$) decomposition. The results shown in Fig. 4, however, led us to the conclusion that there is a direct link between the low-spin methemoglobin–hydroxylamine complex and the hemoglobin–nitric oxide adduct. The gradual disappearance of the former (maximum intensity at *ca.* 2 min) is accompanied by a concomitant increase of the latter (maximum intensity at *ca.* 45 min). We assume that methemoglobin formed in the first step of the reaction sequence reacts with excess hydroxylamine thereby forming the methemoglobin–hydroxylamine adduct. This complex would then be slowly oxidized to the hemoglobin–nitric oxide complex. We cannot say, however, whether additional ESR-silent intermediates are involved. A possible non-radical intermediate could be hydrogen peroxide, formed by two-electron reduction of the hemoglobin-bound dioxygen molecule in the first step of the reaction sequence, where we assume a concomitant formation of the $NH_2O^{\cdot}$ radical and methemoglobin. In this case the stoichiometry would be as follows:

$$HbO_2 + NH_2OH + H^+ \longrightarrow \text{MetHb} + NH_2O^{\cdot} + H_2O_2.$$

We have no evidence for its formation since several difficulties are encountered while trying to detect it using conventional methods. First, the effect of catalase on the reaction rate, a standard test for the involvement of hydrogen peroxide [13], cannot be used in the presence of hydroxylamine, which forms a catalytically inactive complex with catalase [14].

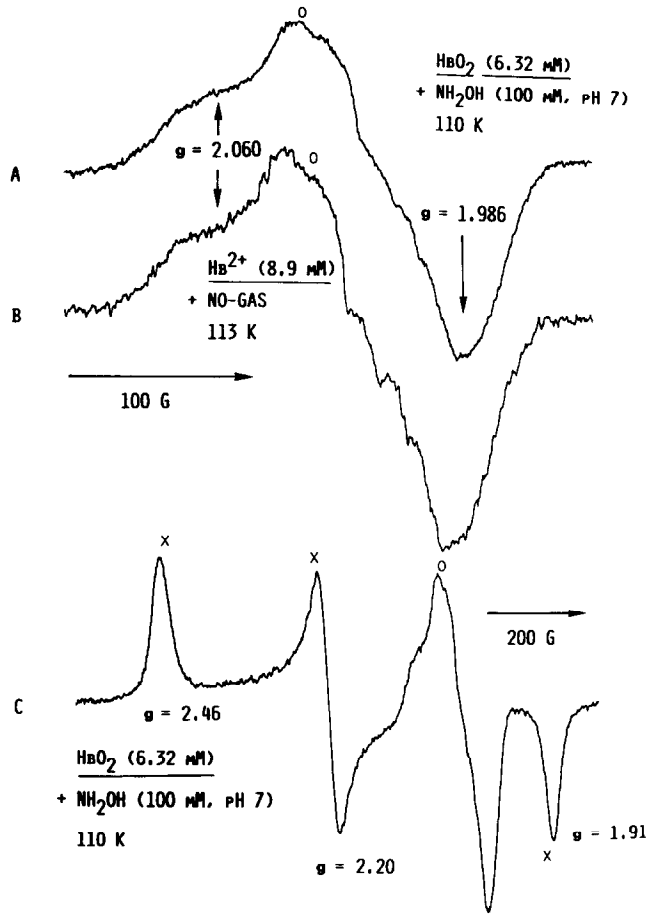

Fig. 3. ESR spectra of the hemoglobin–nitric oxide and the methemoglobin–hydroxylamine complexes. (A) The incubation mixture contained oxyhemoglobin (3.16 mM) and hydroxylamine (50 mM, pH 7). The spectrum was recorded at 110°K. The spectrometer settings were: scan range, 400 G; modulation amplitude, 1 G; receiver gain, 5×10^5; microwave power, 20 mW; time constant, 0.5 sec; scan rate, 48 G/min. (B) The incubation mixture contained deoxyhemoglobin (8.9 mM) and nitric oxide gas (saturated solution at room temperature). The spectrum was recorded at 113°K. The spectrometer settings were: scan range, 400 G; modulation amplitude, 1.25 G; receiver gain, 2.5×10^6; microwave power, 0.25 mW; time constant, 2 sec; scan rate, 24 G/min. (C) The hemoglobin–nitric oxide complex ("O") and the methemoglobin–hydroxylamine complex ("×"). The incubation mixture was the same as in (A). The spectrum was recorded at 110°K. The spectrometer settings were: scan range, 1500 G; modulation amplitude, 1 G; receiver gain, 5×10^5; microwave power, 20 mW; time constant, 0.5 sec; scan rate, 180 G/min.

Second, if hydrogen peroxide were formed close to the hemoglobin moiety, as can be expected, it would immediately react with the heme iron with concomitant formation of ferryl iron as reported by Shiga and Imaizumi [15]. The ESR absorption of iron exhibiting a g-value of g = 2.00 [15] would interfere strongly with the hemoglobin–nitric oxide absorption in the same region so that the expected concentrations cannot be detected, as concluded from preliminary tests where we added small amounts of hydrogen peroxide to the reaction medium (data not shown). We neither could detect superoxide nor hydroxyl radicals in our preliminary spin trapping experiments but it could also be that the spin trap does not get fast enough to the radical formation site and additional problems could arise from the rapid degradation of the spin trap itself. We have work in progress to circumvent this problem.

Putting all this together we suggest the following reaction sequence for the hydroxylamine-induced methemoglobin formation:

$$HbO_2 + NH_2OH + H^+ \longrightarrow NH_2O^{\cdot} + \text{MetHb} + H_2O_2 \quad (1)$$

$$2\,NH_2O^{\cdot} \longrightarrow N_2 + 2\,H_2O \quad (2)$$

$$\text{MetHb} + NH_2OH \longrightarrow \text{MetHb–}NH_2OH \quad (3)$$

$$\text{MetHb–}NH_2OH + 1/2\,O_2 \longrightarrow Hb^{2+}NO + H_2O + H^+. \quad (4)$$

In addition to the above-mentioned hemoglobin adducts, adventitious iron ions play a role in this context. We could show that an iron complex of the type $Fe(NO)_2X_2$ is formed as a byproduct, X representing variable anions such as phosphate or

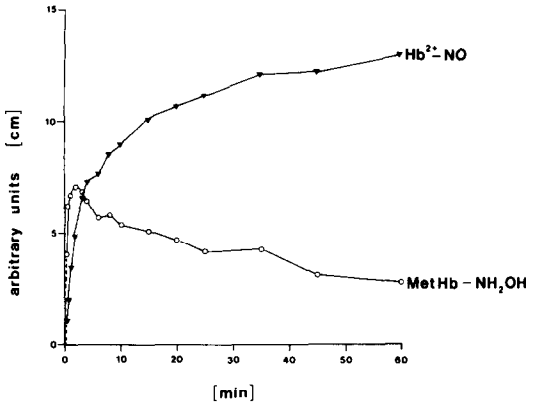

Fig. 4. Time course of the reaction between oxyhemoglobin (0.34 mM) and hydroxylamine (50 mM, pH 7.4). The reaction temperature was 25°. The samples were frozen at different intervals and their ESR spectra were recorded at 77°K. The line marked "▼" represents the Hb–NO complex and the line marked "○" the MetHb–NH_2OH adduct. The intensities represent the height of the most intense peak in arbitrary units.

hydroxide. Although this complex is fairly stable at neutral pH [10], we could not see it in a stationary system because its ESR spectrum overlaps with the far more intense signal of the $Hb^{2+}NO$ complex which exhibits nearly the same g-value. The formation of this complex was completely prevented by the iron chelator DETAPAC and we could show that this did not affect the formation of the other paramagnetic species. This indicates that the formation of this iron complex can be considered as a side reaction which is not involved in the reaction sequence shown above. The origin of the iron ions leading to the $Fe(NO)_2X_2$ complex most probably results from degradation of the hemoglobin moiety [16].

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REFERENCES

1. Bazylinski DA, Arkowitz RA and Hollocher TC, Decomposition of hydroxylamine by hemoglobin. *Arch Biochem Biophys* **259**: 520–526, 1987.
2. Cranston RD and Smith RP, Some aspects of the reactions between hydroxylamine and hemoglobin derivatives. *Pharmacol Exp Ther* **177**: 440–446, 1971.
3. Tomoda A, Matsukawa S, Takeshita M and Yoneyama Y, Opposite effect of organic phosphates on hemoglobin oxidation by hydroxylamine under aerobic and anaerobic conditions. *J Biol Chem* **252**: 6105–6107, 1977.
4. Eyer P, Hertle H, Kiese M and Klein G, Kinetics of ferrihemoglobin formation by some reducing agents, and the role of hydrogen peroxide. *Mol Pharmacol* **11**: 326–334, 1975.
5. Nohl H and Hegner D, Evidence for the existence of catalase in the matrix space of rat heart mitochondria. *FEBS Lett* **89**: 126–130, 1978.
6. Nohl H, Hegner D and Summer KH, The mechanism of toxic action of hyperbaric oxygenation on the mitochondria of rat heart cells. *Biochem Pharmacol* **30**: 1753–1757, 1981.
7. Grisk A, *Praktikum der Pharmakologie und Toxikologie*. VEB Gustav Fischer Verlag, Jena, 1969.
8. Gutch CJW and Waters WA, The electron spin resonance spectra of some hydroxylamine free radicals. *J Chem Soc* **1965**: 751–755, 1965.
9. Adams JQ, Nicksic SW and Thomas JR, Paramagnetic resonance of alkyl nitroxides. *J Chem Phys* **45**: 654–661, 1966.
10. McDonald CC, Phillips WS and Mower HF, An electron spin resonance study of some complexes of iron, nitric oxide, and anionic ligands. *J Am Chem Soc* **87**: 3319–3326, 1965.
11. Kon H, Paramagnetic resonance study of nitric oxide hemoglobin. *J Biol Chem* **234**: 4350–4357, 1968.
12. Jindal VK, Agrawal MC and Mushran SP, Mechanism of the oxidation of hydroxylamine by ferricyanide. *J Chem Soc A* **1970**: 2060–2062, 1970.
13. Aebi H, Katalase. In: *Methoden der Enzymatischen Analyse* (Ed. Bergmeyer HU), pp. 713–724. Verlag Chemie, Weinheim, 1974.
14. Nicholls P and Schönbaum GR, Catalases. In: *Enzymes* (Eds. Boyer PD, Lardy H and Myrbäck K), Vol. 8, pp. 147–225. Academic Press, New York, 1963.
15. Shiga T and Imaizumi K, Electron spin resonance study on peroxidase- and oxidase-reactions of horseradish peroxidase and methemoglobin. *Arch Biochem Biophys* **167**: 469–479, 1975.
16. Gutteridge JMC, Iron promoters of the Fenton reaction and lipid peroxidation can be released from haemoglobin by peroxides. *FEBS Lett* **201**: 291–295, 1986.