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Reinvestigation of the Oxidation Properties of Nitroxides

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The rates of reduction of the two amino-nitroxides, 3-amino-2,2,5,5-tetramethylpyrrolidine-1-oxyl (*I*) and 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (*II*) were compared. Reducing agents, β-mercaptoethanol and sodium ascorbate, were freshly prepared in the presence of nitrogen or air, in a buffer consisting of 0.1 M Na-cacodylate, 0.01 M NaCl, pH 7.

Similar functions of the ESR signal decrease with time were detected for *I* and *II* with both reducing agents under nitrogen when the ratio of nitroxide molarity *versus* reducing agent molarity was kept the same for a given set of experiments. However, a significant difference in the ESR signal decrease with time was observed between *I* and *II* with ascorbate in the presence of air. Reproducible time dependence of the ESR signal decay required the use of freshly prepared reducing solutions.

Our results strongly suggest that at least amino substituted five and six-membered ring nitroxides oxidize electron donors at the same rate. The apparent greater stability of *I versus II* seems to be due to the slower reoxidation rate of the hydroxylamine of *II* as compared to the hydroxylamine of *I*. Thus, under *in vivo* conditions, *I* and *II* will become ESR silent at the same rate unless the tissue is not under anaerobic conditions.

INTRODUCTION

Stable nitroxide free radicals have been used extensively as spin labels in the study of molecular transport in membranes, in tissue homogenates,[1-8] *in vivo* ESR studies[9,10] and recently as contrast-enhancing agents for nuclear magnetic resonance imaging (MRI).[1,11]

These nitroxides are known to be reduced by thiols, ascorbate and cell cultures. The hydroxylamines that result from such reduction are good electron donors for mild oxidants. Studies of nitroxide reduction by ascorbate and tissue homogenates have sug-

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gested that five-membered ring nitroxides are substantially more resistant towards reduction than six-membered ring nitroxides. The influence of oxygen on the ascorbate spin probe reaction was described[12] and an important role of the superoxide radical in the reoxidation of hydroxylamine was suggested.[12,13] However, the interplay between the ring structure, reducing mechanisms and the oxygen in the reaction of nitroxides with reducing agents is not well understood.

In this communication, we have compared the reduction rates of the amino-nitroxides *I* and *II* with a freshly prepared β-mercaptoethanol and sodium ascorbate in the presence of air or nitrogen.

EXPERIMENTAL

3-amino-2,2,5,5-tetramethylpyrrolidine-N-oxyl (*I*) (Kodak) and 4-amino-2,2,6,6-tetramethylpiperidine-N-oxyl (*II*) (Aldrich) were used without further purification. The reducing agents, β-mercaptoethanol (Sigma) and sodium salt of L-ascorbic acid (Sigma) were used as such. For all measurements, a buffer consisting of 0.1 M Na-cacodylate, 0.01 M NaCl, at pH 7, with or without EDTA, was used. Solutions of both reducing agents, ascorbate and β-mercaptoethanol in buffer were prepared immediately before the ESR measurements. The ESR spectra were recorded with a BRUKER ESP-300 spectrometer.

For anaerobic conditions, the buffer was bubbled with nitrogen for about 10 hours. The selected reactant concentrations were then prepared and additionally blown with nitrogen for about a minute. The ESR signal decrease with time of nitroxides was measured by determining the ratio of the amplitude of one nitroxide line and one manganese marker line. The ESR signal decrease with time was represented as percent of the ESR signal left. 100 % of the ESR signal was obtained by addition of an equivalent volume of buffer instead of reducing agent.

RESULTS

Reaction with β-Mercaptoethanol

Figure 1 shows the ESR signal decrease with time of spin label *I* in the presence of various excesses of β-mercaptoethanol in the air.

Under anaerobic conditions, the ESR signal decrease with time was faster, as it is shown in Figures 2 and 3. β-Mercaptoethanol was used in an 100% excess, which gave pseudo first order rates under anaerobic conditions. The rate of the ESR signal decrease with time was significantly dependent on the age of the β-mercaptoethanol dilution with buffer (Figure 2), in buffers with and without EDTA.

Within the experimental error, there is no difference in the function of the ESR signal decrease with time for five (*I*) and six-membered ring (*II*) nitroxides, *i.e.* both spin labels show a much faster ESR signal decrease with time under anaerobic conditions (Figure 3) than under aerobic conditions (Figure 4). Thus, oxygen has the effect of similarly slowing down the reduction rates of both spin labels.

Reaction with Sodium Ascorbate

For all measurements 100 fold excess of sodium ascorbate over nitroxide was used.

In Figure 5 the ESR signal decrease with time of the spin label *I* is compared with that of spin label *II* in air. A significantly faster decrease of the ESR signal with time was observed for the six-membered than for the five-membered ring nitroxide.

On the other hand, if the reaction was performed under anaerobic conditions, *i.e.* in the presence of nitrogen, the decrease of ESR signal with time was equally fast for

TABLE I

Half-life, $t_{1/2}$, (seconds) of the ESR signal of nitroxides in the reaction with reducing agents

reducing agent	nitroxide			
	five-membered ring (*I*)		six-membered ring (*II*)	
	O_2	N_2	O_2	N_2
β-mercaptoethanol	3600	1080–1800	3600	1080–1800
sodium ascorbate	120–300	<5	<10	<5

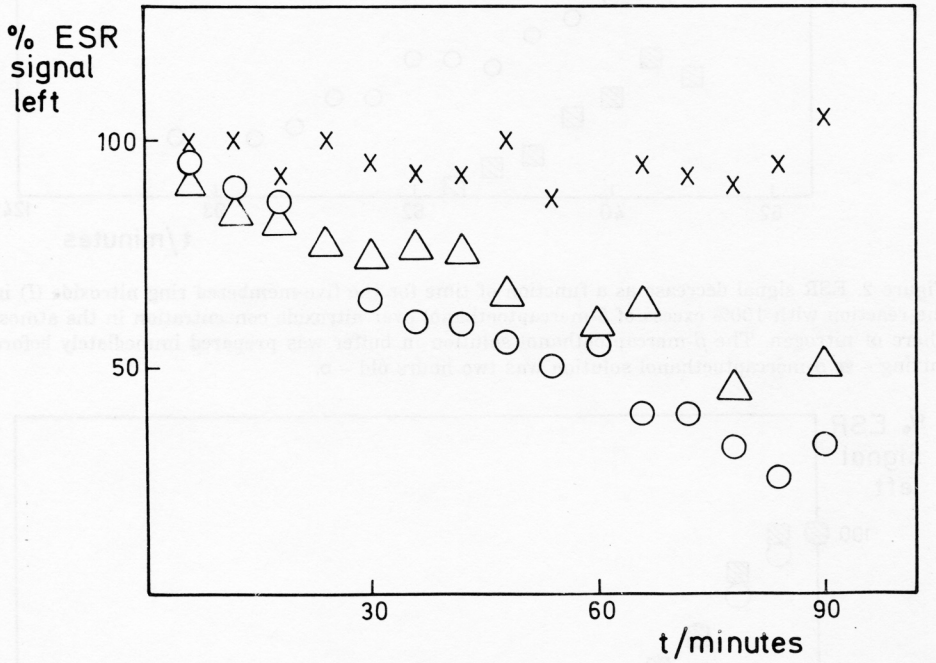

Figure 1. ESR signal decrease as a function of time for the five-membered ring nitroxide (*I*) in the reaction with β-mercaptoethanol in the presence of air: x – 10% excess of β-mercaptoethanol; Δ – 100% excess of β-mercaptoethanol; o – 1000% excess of β-mercaptoethanol.

both spin labels. Under nitrogen, the ESR signal decrease with time was so fast that the results were collected in the time sweep mode. For this purpose, the magnetic field was positioned on the low field line at the signal maximum, and 60 seconds time sweeps were carried out. The time interval between the addition of the ascorbate to nitroxide and the start of the collection of the data was typically 13 seconds.

In Figure 6a, the ESR signal decrease with time of the five-membered ring (*I*) is shown and Figure 6b displays the data for the six-membered ring (*II*).

Table I shows half-life ($t_{1/2}$) values of the ESR signal for nitroxides in the reaction with both reducing agents. $t_{1/2}$ is defined as the time required for the remaining 50% of the ESR signal.

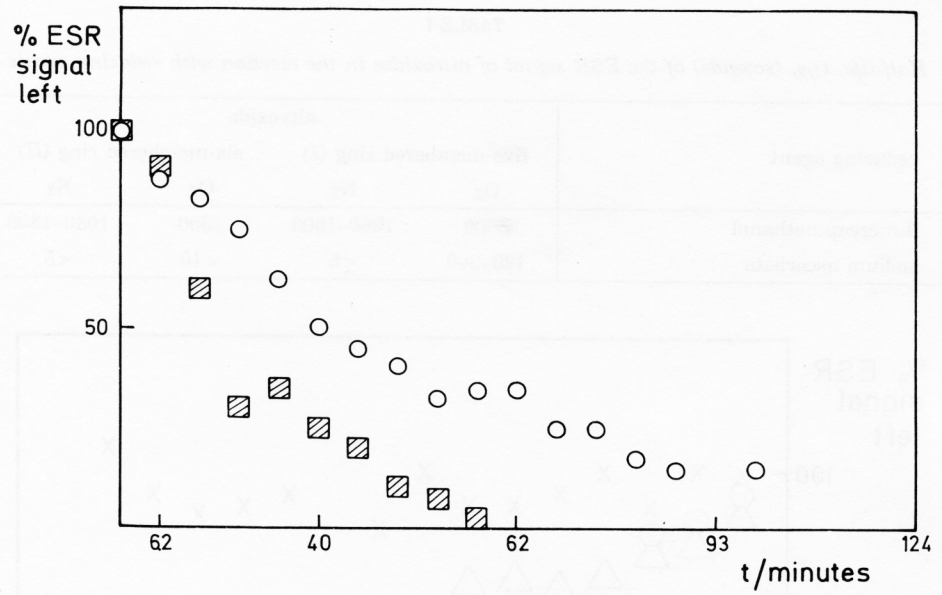

Figure 2. ESR signal decrease as a function of time for the five-membered ring nitroxide (*I*) in the reaction with 100% excess of β-mercaptoethanol over nitroxide concentration in the atmosphere of nitrogen. The β-mercaptoethanol solution in buffer was prepared immediately before mixing – ■; β-mercaptoethanol solution was two hours old – o.

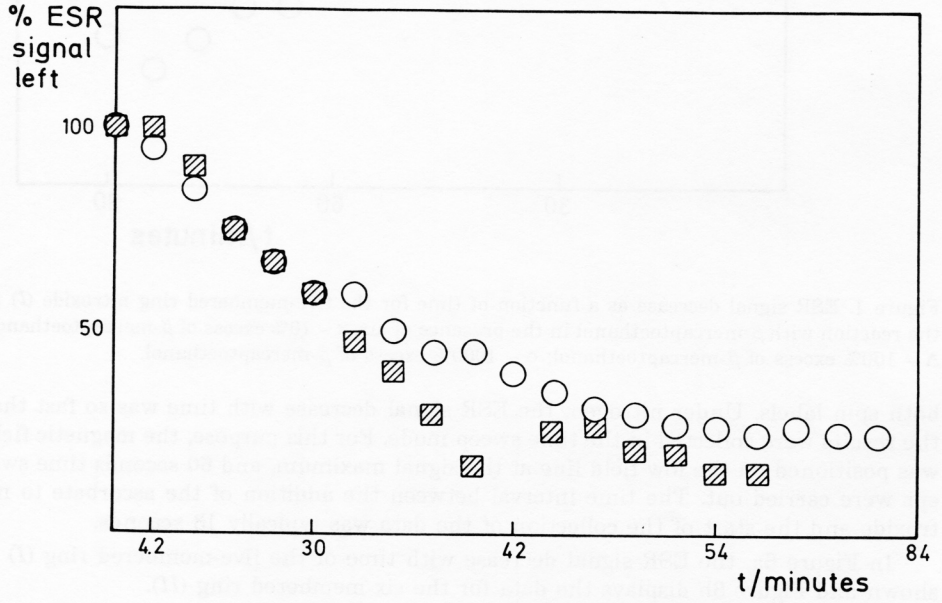

Figure 3. ESR signal decrease as a function of time for the five-membered ring nitroxide (*I*) – ■, and the six-membered ring nitroxide (*II*) – o, in the reaction of freshly prepared β-mercaptoethanol (100% excess over nitroxide concentration) in a nitrogen atmosphere.

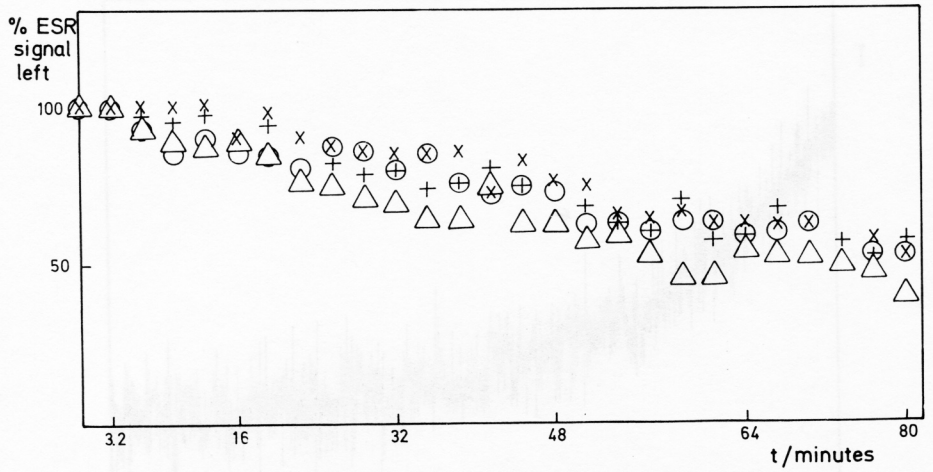

Figure 4. ESR signal decrease as a function of time for the five-membered ring nitroxide (*I*) – x and + ; and the six-membered ring nitroxide (*II*) – Δ and o ; in the reaction with freshly prepared β-mercaptoethanol (100% excess over nitroxide concentration) in air. For each nitroxide, two experiments are shown in order to differentiate the experimental error from the difference between the reactions of five- and six-membered ring nitroxides.

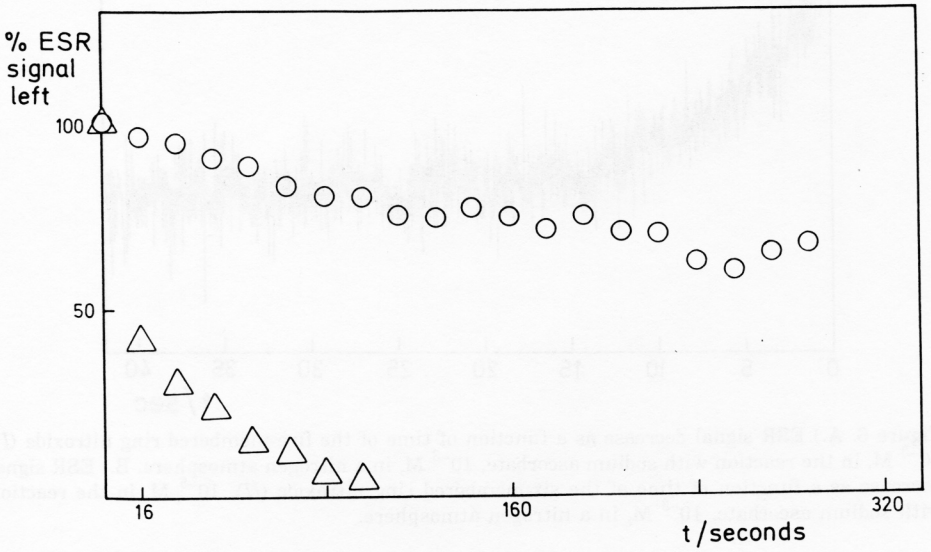

Figure 5. ESR signal decrease as a function of time of the five-membered ring nitroxide – o; and of the six-membered ring nitroxide (*II*) – Δ in the reaction with sodium ascorbate (100% excess of ascorbate over the nitroxide concentration) in air.

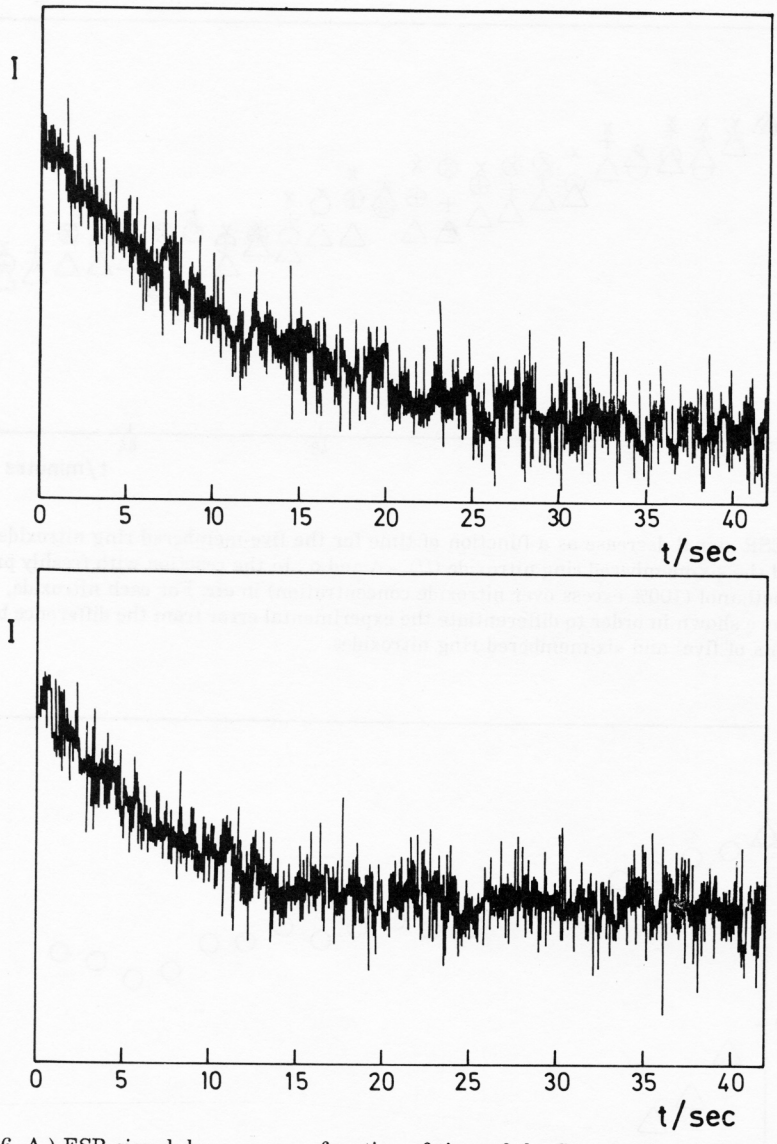

Figure 6. A.) ESR signal decrease as a function of time of the five-membered ring nitroxide (*I*), 10^{-5} M, in the reaction with sodium ascorbate, 10^{-3} M, in a nitrogen atmosphere. B.) ESR signal decrease as a function of time of the six-membered ring nitroxide (*II*), 10^{-5} M, in the reaction with sodium ascorbate, 10^{-3} M, in a nitrogen atmosphere.

DISCUSSION

Numerous results reported in the literature suggested that the five-membered ring nitroxides were more resistant towards reduction by –SH reducing agents, ascorbate and tissue homogenates,[2,7,15] and oxygen was found to slow down the reduction rates of nitroxides.[2] Also, the reaction of hydroxylamine with oxygen was described,[14] and

it was proposed that it could be used for the production of superoxide ion with the production of nitroxide radical.

We have reexamined the reaction of amino-derivatives of six- and five-membered ring nitroxides with sodium ascorbate and with β-mercaptoethanol in the presence of air and in the presence of nitrogen, *i.e.* under oxygen devoid conditions. If the reactions are carried out under oxygen devoid conditions, there is no difference in the rates of reduction of amino-substituted five- and six-membered ring nitroxides with both reducing agents. This is in accordance with the results reported by Schara and coworkers for oxazolidine and piperidine nitroxides,[13] and with the identical half-wave reduction potentials of five- and six-membered ring nitroxides.[16]

With ascorbate, the presence of air had a profound effect on slowing down the ESR signal decay of the five-membered ring nitroxide. However, no such effect was observed with the six-membered ring nitroxide.

Most of the literature data on nitroxide reaction with ascorbate were obtained in the presence of air.[1,17,18] It is well known that ascorbyl radical is effective in lipid peroxidation[19] *i.e.* it reacts with O_2 to produce O_2^-, the superoxide radical. Reaction of O_2^- radical with nitroxide depends on the ratio of the rates: rate of nitroxide reduction to hydroxylamine and the rate of the oxidation of corresponding hydroxylamine to nitroxide.[20] It was found recently[20] that six-membered ring nitroxides react with superoxide radical about two orders of magnitude faster than the corresponding five-membered ring nitroxides. This is in accordance with the finding that the five-membered ring hydroxylamine is oxidized by air ten times faster than the six-membered ring hydroxylamine.[21] Our results suggest that the apparent »resistance« of the five membered ring nitroxides towards ESR signal loss has its origin in the reaction of the resulting hydroxylamine with O_2^-. In the case of the six membered ring hydroxylamine, this reaction is less efficient. β-Mercaptoethanol is a weaker reducing agent than ascorbate (Table I). Nitroxide mediated oxidation of thiols to sulfonic acid was first described by Morrisett and Drot,[22] and was extensively studied later.[23,24] It was demonstrated that the simultaneous presence of O_2^-, thiol and nitroxide results in the reduction of the nitroxide to its corresponding hydroxylamine,[25] *i.e.* the presence of thiols makes the reduction of nitroxides by superoxide radical a more effective process if superoxide and thiol concentrations are similar.[25] This process is nitroxide specific as a consequence of the superoxide nitroxide reaction specificity. In our experiments, superoxide radical could be formed in the autooxidation of thiols in the presence of air. On the other hand, it was shown that autooxidation of thiols is pH dependent and is an extremely inefficient process at pH 7, even in the presence of 10^{-4} molar O_2.[24] Thus, under the conditions of our experiment, the specificity of nitroxide reaction with superoxide radical in the presence of thiols could not be observed. The presence of oxygen had equal effect on slowing down the reaction with five- and six-membered ring nitroxides. Assuming the model proposed by Schara and collaborators,[13] *i.e.* that nitroxides act as mediators for the redox reactions between reducing agent and oxygen, we propose that the redox process with a half-life, $t_{1/2}$, of about 60 minutes, at pH 7, between nitroxide, β-mercaptoethanol and oxygen does not express a catalytic activity difference between five- and six-membered ring nitroxides. The »resistance« to the ESR signal loss of nitroxide in the presence of oxygen seems to depend on the catalytic properties of nitroxide itself, but also on the reaction specificities between the reducing agent and oxygen. Each redox system has its reaction rate specificities, and the behaviour of one nitroxide in one redox system may be different in another redox system.

In summary, under pseudo-first order reaction conditions, a five-membered ring nitroxide can display an apparently greater stability than a six membered ring nitroxide in certain coupled redox systems, such as ascorbate and oxygen.

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SAŽETAK

Ponovno ispitivanje oksidacijskih svojstava nitroksida

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Uspoređivane su brzine redukcije dvaju amino-nitroksida, 3-amino-2,2,5,5-tetrametilpirolidine-1-oksil (*I*) i 4-amino-2,2,6,6-tetrametilpiperidine-1-oksil (*II*). Upotrijebljeni su redukcijski agensi, svježe pripremljen β-merkaptoetanol ili natrij-askorbat u atmosferi dušika ili zraka, u puferu 0.1 M Na-kakodilat, 0.01 M NaCl kod pH = 7.

U atmosferi dušika određene su slične funkcije opadanja ESR signala s vremenom za *I* i *II* s oba redukcijska agensa, za isti omjer koncentracije nitroksida prema redukcijskom agensu. Reproducibilna ovisnost opadanja ESR signala s vremenom dobivena je upotrebom svježe pripremljenih otopina reducensa.

Rezultati sugeriraju da amino-supstituirani nitroksidi u peteročlanom i u šesteročlanom prstenu oksidiraju elektron-donore jednakom brzinom. Prividno veća stabilnost *I* s obzirom na *II* čini se da potječe od manje brzine reoksidacije hidroksilamina *II* u usporedbi s hidroksilaminom *I*. Zato u *in vivo* uvjetima treba očekivati da će *I* i *II* postati istom brzinom »ESR-utišani«, osim ako tkivo nije pod anaerobnim uvjetima.