Reduction of β-phosphorylated cyclic aminoxyl radicals by flavins: an EPR kinetic study †

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A series of stable β-phosphorylated aminoxyl radicals has been tested towards their resistance to photoreduced flavin reduction in a pH 7.4 phosphate buffer. Their decay kinetics, monitored by EPR spectroscopy, have been compared to those of three non-phosphorylated reference compounds. In each case, both a second-order reduction and a first-order aminoxyl decay were found to contribute to the EPR signal loss. On the basis of only the second-order process, a classification of the aminoxyl radicals has been established as a function of their ease of reduction by the two flavins tested.

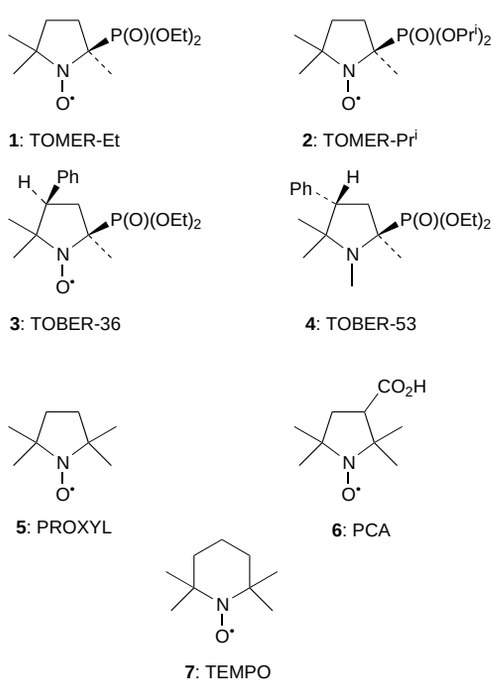
The potential applications of stable aminoxyl free radicals became increasingly important with the observation that these compounds, which have been widely used *in vitro*, could also be employed successfully for various *in vivo* studies.[1] For example, they have been used as EPR spin probes to study biomembrane properties,[2] or to determine oxygen level in various cellular microenvironments.[3] Moreover, they were found to be valuable contrast-enhancing agents for magnetic resonance imaging,[4] and they also play a key-role in EPR imaging when used in intact organs or in whole animals.[5] These aminoxyls have been shown to be transformed into EPR-silent hydroxylamine by a large range of reducing agents, such as ascorbate or thiols,[6] and this reduction should be regarded as a serious limitation in a lot of *in vivo* applications. This is the reason why both their *in vivo* and *in vitro* reduction by various models of bioreducing agents have been widely studied.[7]

The variety of stable aminoxyl applications resulted in the necessity to dispose of a large range of compounds showing various properties, particularly concerning their lipophilicity, their EPR parameters and their decay rates in various environments.[8] In this field, a new series of stable β-phosphorylated cyclic aminoxyls has been elaborated in our laboratory a few years ago.[9] All these pyrrolidinoxyl radicals exhibited EPR spectra showing a large hyperfine splitting constant (hfsc) with the phosphorus nucleus ranging from 3.6 to 5.5 mT, which was found to be very sensitive to the five-membered ring conformation. Because of this strong coupling, these β-phosphorylated aminoxyls could be valuable tools for example in the study of biomembrane properties. Furthermore, the presence of a dialkoxyphosphoryl group on the ring could result in interesting modifications of the metabolism of these compounds or in their biodistribution. Anyway, before using these new spin labels *in vivo*, it is of prime importance to evaluate their resistance to the action of various bioreducing agent models, in order to have a notion of their lifetime in biological systems. Thus, their reduction rate by ascorbate has been previously evaluated in pH 7.4 phosphate buffer.[10] In order to proceed with this study, we have examined the resistance of these β-phosphorylated compounds towards the action of photoreduced flavins.

Results

When irradiated with visible light in the presence of an electron donor, oxidised flavins are known to undergo a two-step reduction, yielding a dihydro form of the flavins.[11,12] These photoreduced compounds can then react efficiently with many chemical or biochemical molecules, such as triplet oxygen,[13] and this reaction has been frequently employed to generate superoxide in aqueous media.[14,15] A few studies have also been devoted to the transformation of aminoxyls into EPR-silent hydroxylamines by photoreduced flavins.[12,16]

In our study, two flavins, riboflavin and flavin mononucleotide (FMN) considered as bioreducing agent models, were employed to reduce a series of aminoxyls. All the experiments were conducted in a pH 7.4 phosphate buffer and in the absence of oxygen to avoid reoxidation of the hydroxylamine formed. The seven aminoxyls, the structures of which are shown below, were studied. They can be divided into two groups: four β-phosphorylated compounds, *i.e.* 2-diethoxyphosphoryl-2,5,5-trimethylpyrrolidin-1-oxyl **1** (TOMER-Et), 2-diisopropyloxyphosphoryl-2,5,5-trimethylpyrrolidin-1-oxyl **2** (TOMER-Pr^i), *r*-2-diethoxyphosphoryl-*c*-4-phenyl-2,5,5-trimethylpyrrolidin-1-oxyl **3** (TOBER-36) and *r*-2-diethoxyphosphoryl-*t*-4-phenyl-2,5,5-trimethylpyrrolidin-1-oxyl **4** (TOBER-53), and three non-phosphorylated probes, *i.e.* 2,2,5,5-tetramethylpyrrolidin-1-oxyl **5** (Proxyl), 3-carboxy-



1: TOMER-Et **2**: TOMER-Pr^i **3**: TOBER-36 **4**: TOBER-53 **5**: PROXYL **6**: PCA **7**: TEMPO

† Presented at the 30th International Meeting of the Electron Spin Resonance Group of the RSC, University of Lancaster, 6–10th April 1997.

Table 1 EPR hyperfine splitting constants of aminoxyls **1**–**7** in 0.1 mol dm^{-3} phosphate buffer, pH 7.4

Aminoxyl	a_N/mT	a_P/mT
Proxyl **5**	1.63	—
PCA **6**	1.62	—
TOMER-Pr^i **2**	1.54	4.76
TOMER-Et **1**	1.53	4.79
TOBER-36 **3**	1.52	3.64
TOBER-53 **4**	1.52	5.50
TEMPO **7**	1.72	—

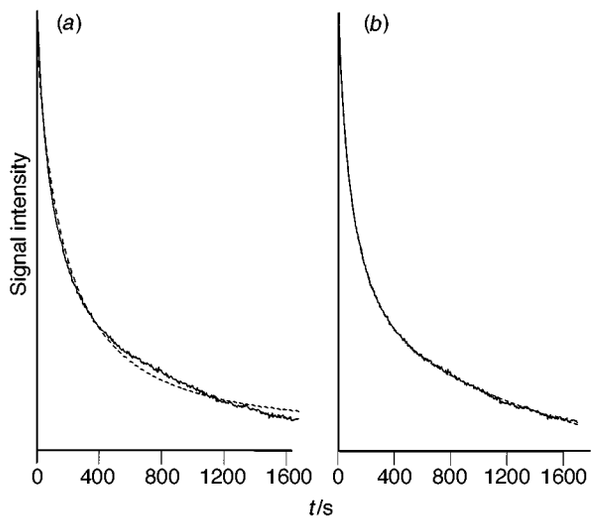


Fig. 1 Decay kinetic of TOMER-Pr^i **2** recorded in the presence of photoreduced riboflavin in a 0.1 mol dm^{-3} pH 7.4 phosphate buffer. Modelling of the decay (- - - -) has been achieved using (*a*) eqn. (1), with $k_{red} = 107$ dm^3 mol^{-1} s^{-1}, or (*b*) eqn. (2), with $k_{des} = 4 \times 10^{-4}$ s^{-1} and $k_{red} = 137$ dm^3 mol^{-1} s^{-1}.

2,2,5,5-tetramethylpyrrolidin-1-oxyl **6** (PCA) and 2,2,6,6-tetramethylpiperidin-1-oxyl **7** (TEMPO).

The EPR parameters of the aminoxyl spectra recorded in phosphate buffer, *i.e.* the hfscs for the nitrogen and the β-phosphorus, have been listed in Table 1. In each kinetic experiment, the medium containing the aminoxyl, an oxidised flavin and diethylenetriaminetetraacetic acid (DTPA) was first irradiated with visible light to generate photoreduced flavin, as described in the Experimental section. After termination of the illumination, the aminoxyl decay was followed by recording the EPR signal at a fixed field corresponding to the first low-field peak of the spectrum.

The 14 experimental kinetic curves, corresponding to the signal loss of aminoxyls **1**–**7** in the presence of each one of the two flavins, were first modelled considering only a simple second-order reduction. The rate of aminoxyl decay was then given by eqn. (1) in which $[RR'NO^{\bullet}]$ and $[Fl_{red}]$ represent the aminoxyl

$$-d[RR'NO^{\bullet}]/dt = k_{red}[RR'NO^{\bullet}][Fl_{red}] \quad (1)$$

and the photoreduced flavin concentrations, respectively, and k_{red} the second-order rate constant. However both the standard deviation between experimental and calculated curves, and the uncertainty in the determination of k_{red} were found much too high (*e.g.* a 30% error was evaluated for k_{red} in the case of the reduction of **5** by riboflavin). Furthermore, a second-order reduction process was not appropriate to model experimental TEMPO decay curves. Fig. 1(*a*), in which both the experimental curve of TOMER-Pr^i decay in the presence of reduced riboflavin and the curve calculated using eqn. (1) have been plotted, clearly shows that a single second-order reduction does not correctly reproduce the experimental decay kinetics.

Another model, described in Scheme 1, was then examined.

Table 2 Rate constants determined for the first-order decay of the aminoxyls **1**–**7** in the presence of either riboflavin ($k_{1,rib}$) or FMN ($k_{1,FMN}$). The values have been determined by computer simulation of experimental decay curves using eqn. (2).

Aminoxyl	$k_{1,rib}/10^{-4}$ s^{-1}	$k_{1,FMN}/10^{-4}$ s^{-1}
Proxyl **5**	0–8	0–0.5
PCA **6**	0–8 × 10^{-3}	0–0.4
TOMER-Pr^i **2**	4.0 ± 0.1	1.0 ± 0.1
TOMER-Et **1**	0.3 ± 0.08	1.2 ± 0.1
TOBER-36 **3**	2.14 ± 0.04	1.06 ± 0.03
TOBER-53 **4**	0.92 ± 0.4	1.3 ± 0.03
TEMPO **7**	3.0 ± 0.1	3.4 ± 0.1

Table 3 Second-order rate constants determined for the reduction of aminoxyls **1**–**7** by either riboflavin ($k_{red,rib}$) or FMN ($k_{red,FMN}$). The values have been determined by computer simulation of experimental decay curves using eqn. (2).

Aminoxyl	$k_{red,rib}$/ dm^3 mol^{-1} s^{-1}	$k_{red,FMN}$/ dm^3 mol^{-1} s^{-1}
Proxyl **5**	29 ± 3	52 ± 4
PCA **6**	63 ± 3	77 ± 1
TOMER-Pr^i **2**	137 ± 2.5	233 ± 10
TOMER-Et **1**	251 ± 3	426 ± 8
TOBER-36 **3**	323 ± 10	608 ± 10
TOBER-53 **4**	422 ± 16	718 ± 14
TEMPO **7**	503 ± 29	811 ± 10

$$RR'NO^{\bullet} \xrightarrow{k_1} X_{(diamagnetic)}$$

$$RR'NO^{\bullet} + Fl_{red} \xrightarrow{k_{red}} RR'NOH + Fl_{ox}$$

Scheme 1 Model proposed for the description of the aminoxyl **1**–**7** experimental decay observed in the presence of photoreduced flavins in phosphate buffer. $RR'NO^{\bullet}$ represents the aminoxyl, RR′NOH the corresponding hydroxylamine, X an unidentified diamagnetic product, Fl_{red} and Fl_{ox} the reduced and oxidised flavin, respectively; k_1 and k_{red} correspond to the first- and second-order rate constants, respectively.

In this case, a first-order decay was considered to contribute to the aminoxyl decay together with its reduction by the flavin.

The corresponding rate is thus given by eqn. (2) in which

$$-d[RR'NO^{\bullet}]/dt = k_1[RR'NO^{\bullet}] + k_{red}[RR'NO^{\bullet}][Fl_{red}] \quad (2)$$

$[RR'NO^{\bullet}]$ and $[Fl_{red}]$ represent the aminoxyl and the photoreduced flavin concentrations, respectively, k_1 being the first-order rate constant of the aminoxyl decay and k_{red} the second-order rate constant of the aminoxyl reduction by flavin. Using this second model, simulation of each one of the 14 experimental curves could be successfully achieved, yielding to standard deviation values always in the range of the signal-to-noise ratio. The values thus determined for the two rate constants k_1 and k_{red} have been listed in Tables 2 and 3, respectively.

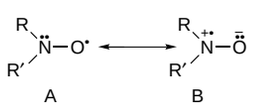
Fig. 1(*b*) shows the experimental curve of TOMER-Pr^i decay in the presence of riboflavin which has been modelled using eqn. (2); it appears that experimental and calculated curves are perfectly superimposed. This excellent fit between the two curves clearly illustrates the amelioration obtained in the modelling by taking into account a first-order decay process in addition to the second-order reduction, and supports the validity of the model proposed.

Discussion

Considering only the values indicated for k_{red}, that is, taking into account only the second-order reduction process itself, the seven aminoxyls tested can be compared as a function of their resistance to the reduction by flavins. The classification thus obtained appears in Table 3, in which aminoxyls **1**–**7** have been listed from the more to the less resistant to the reduction. This

classification was found to be independent of the flavin, although FMN was a *ca.* 1.5 times stronger reducing agent than riboflavin in our experimental conditions. Thus, TEMPO is clearly the most easily reduced aminoxyl, and this is in accordance with all the results given in the literature which specify that piperidinoxyl compounds are more easily reduced than are pyrrolidinoxyl compounds.[17] It also appeared that the introduction of a β-phosphoryl group on the pyrrolidinyl ring resulted in an increase in the reduction rate of the aminoxyl. Thus, TOMER-Pr^{i} **2** and TOMER-Et **1** were reduced *ca.* 4.5 and 8.5 times more rapidly, respectively, than their non-phosphorylated analogue, Proxyl **5**. The same classification of these aminoxyls was previously observed when they were tested for their resistance to ascorbate reduction,[10] but ascorbate was found to reduce compounds **1**–**6** from 4 to 10 times slower than riboflavin. This result is in accordance with the literature data,[16] which indicate that flavins are much more powerful reducing agents than ascorbate. In addition, the reduction of compounds **1**–**6** by ascorbate has been shown to be reversible in pH 7.4 phosphate buffer,[10] thereby enhancing the persistence of these aminoxyls in the presence of this reducing agent. So we thought that it was important to verify that the aminoxyl reduction by flavins was not reversible. Thus, various aminoxyl reduction experiments have been performed using the conditions described in the Experimental section and followed by EPR spectroscopy. A large amount of oxidised flavin was then added after the loss of *ca.* 66% of the initial EPR signal, and the medium was kept in the dark. No significant increase in the EPR signal was observed, indicating that the oxidised flavins were unable to convert hydroxylamine into aminoxyl.

If the relative reduction rate in the pyrrolidinoxyl series can be considered independent of the reducing agent, it seems to increase while the hfsc a_N decreases (see Table 1). Aminoxyl compounds can be represented by the two mesomeric forms A and B, as indicated in Scheme 2, and the a_N value is expected to



Scheme 2 Representation of the two limit mesomeric forms of an aminoxyl

increase when the B form is favoured. As previously observed,[10] the presence of an electron-withdrawing group, such as a dialkoxyphosphoryl, in the β-position to the aminoxyl function results in favouring the mesomeric form A, in which the unpaired electron is located on the oxygen. This aminoxyl is then a better hydrogen abstracting agent and is more easily reducible to the corresponding hydroxylamine.

However, beside electronic factors, the aminoxyl stereochemistry seems to have a small influence on the second-order decay mechanism. Different reduction rates have been determined for the two diastereoisomeric aminoxyls **3** and **4**, although these two compounds show the same a_N. Thus, TOBER-36 **3** appeared to be *ca.* 1.2 times more resistant than TOBER-53 **4** to the flavin reduction.

Considering now only the first-order decay process, the interpretation of the results obtained in this kinetic study seems to be much more tricky. As can be seen from Table 2, k_1 was most often found in the range of 10^{-4} s^{-1}. However, in the case of Proxyl, for example, the estimation of k_1 was tedious and values ranging from 0 to 8×10^{-4} s^{-1} were found. In general, we observed that the determination of k_1 was all the more difficult as this rate constant was found to depend greatly on experimental conditions, such as the power of the lamp, the irradiation time, or the sample volume irradiated. This could explain the importance of the error in the determination of k_1 (see Table 2). Since the meaning of the first-order decay remained unclear, we thought that it was necessary to verify that as aminoxyl destruction really occurred during the spin loss observed. Using potassium ferricyanide, a selective oxidating agent for hydroxylamines, a reoxidation of the hydroxylamine formed during the aminoxyl decay resulted in the recovering of only 65–85% of the initial EPR signal. When H_2O_2 was employed as the oxidating agent, the percentage of the aminoxyl obtained was generally found higher than 85% and could sometimes reach 95%. This clearly indicates on one hand that an irreversible destruction of at least 5% of the aminoxyl occurred, and on the other hand that the photoreduced flavins tested were also able to reduce hydroxylamines in amines. By computer integration of eqn. (2), the proportion of diamagnetic product X formed after 1800 s of aminoxyl decay was found to vary between 6 and 25%, depending on the aminoxyl and the flavin tested. The comparison between these two results indicated that in each case, the product X concentration calculated was slightly higher than the effective proportion of aminoxyl decay. In addition, an EPR signal decrease was observed when an aminoxyl solution containing oxidised flavin was kept in the dark. This aminoxyl decay was found to be pure first-order, and the rate constants thus evaluated were in the range of 10^{-5} s^{-1}, *i.e.* significantly lower than k_1 values indicated in Table 2. The same kind of phenomenon was also observed when a solution containing DTPA and an aminoxyl was irradiated in the absence of flavin. This corroborates the hypothesis that the reduction by flavins was not the only process involved in the aminoxyl decay. However, all these results seem also to indicate that the first-order decay experimentally observed possibly corresponds to several pseudo-first-order processes occurring simultaneously. Actually, it is quite clear that the use of eqn. (2) to model the aminoxyl decay sometimes resulted in a slight over-estimation of the aminoxyl destruction.

A likely hypothesis to explain this result is the following. The reaction of an aminoxyl with a photoreduced dihydroflavin (*i.e.* FlH_2) has been reported to give hydroxylamine and a monohydroflavin (*i.e.* $FlH^{\bullet}$),[11,12] the two forms FlH_2 and $FlH^{\bullet}$ being able to reduce an aminoxyl. In this case, the kinetic model indicated in Scheme 3 would describe the aminoxyl decay. The rate

$$RR'NO^{\bullet} \xrightarrow{k_{des}} X_{(diamagnetic)}$$

$$RR'NO^{\bullet} + FlH_2 \xrightarrow{k_{red}} RR'NOH + FlH^{\bullet}$$

$$RR'NO^{\bullet} + FlH^{\bullet} \xrightarrow{k'_{red}} RR'NOH + Fl_{ox}$$

Scheme 3 Hypothesis for the mechanism of the aminoxyl decay observed in the presence of photoreduced flavins in phosphate buffer. $RR'NO^{\bullet}$ represents the aminoxyl, RR′NOH the corresponding hydroxylamine, X a diamagnetic product, FlH_2, $FlH^{\bullet}$ the dihydro and monohydro reduced forms of the flavin, respectively, Fl_{ox} corresponding to oxidised flavin; k_{des}, k'_{red} and k_{red} correspond to first- and second-order rate constants.

$$-d[RR'NO^{\bullet}]/dt = (k_{des} + k'_{red}[FlH^{\bullet}])[RR'NO^{\bullet}] + k_{red}[RR'NO^{\bullet}][FlH_2] \quad (3)$$

of aminoxyl decay would then be given by eqn. (3) in which k_{des} corresponds to the first-order rate constant of the aminoxyl destruction, k_{red} and k'_{red} being the second-order rate constants of the aminoxyl reduction by the dihydro- and the monohydroflavin, respectively. On the basis of UV–kinetic studies of aminoxyl reduction by flavins, Chan and Bruice[12] have clearly shown that under our experimental conditions (pH < 9), the monohydroflavin $FlH^{\bullet}$ accumulates in the medium as a so-called 'disproportionation dimer' complex, which can also be obtained from the forms FlH_2 and Fl_{ox}, as indicated in Scheme 4. Because of this phenomenon, no simple relation

$$^2FlH^{\bullet} \rightleftharpoons [complex] \rightleftharpoons Fl_{ox} + FlH_2$$

Scheme 4

can be established between the $FlH^{\bullet}$ and FlH_2 concentrations. Thus, a steady-state concentration can be assumed for the monohydroflavin $FlH^{\bullet}$, the aminoxyl reduction by $FlH^{\bullet}$ became pseudo-first-order, and substituting eqn. (2) into eqn. (3), with $[Fl_{red}] = [FlH_2]$, gives eqn. (4).

$$k_1 = k_{des} + k'_{red}[FlH^{\bullet}] \quad (4)$$

In addition, this could explain why the constant k_1 was found to depend greatly on the experimental conditions. Since under these conditions it was impossible to determine the $FlH^{\bullet}$ concentration, we were unable to evaluate separately k'_{red} and k_{des}. In addition, note that the mechanism responsible for the irreversible destruction of the aminoxyl remained unclear. However, this last reaction was much less important than the reduction, which appeared to be the major process responsible for the aminoxyl decay in the presence of photoreduced flavin.

Conclusions

The EPR kinetic study presented in this paper indicates that the aminoxyl signal loss in the presence of photoreduced flavins could be correctly simulated by considering both a second-order reduction and a minor first-order decay.

Although effective aminoxyl destruction was unambiguously shown to occur, the observed rate constant k_1 was probably the sum of several first- or pseudo-first-order constants. In particular, an aminoxyl reduction by the monohydro form of the flavins tested might intervene in the first-order behaviour observed.

Since the second-order reduction appeared to be the major mechanism responsible for the aminoxyl decay studied, the determination of the corresponding rate constant allowed us to classify the different aminoxyls in terms of their resistance to flavin reduction. In the pyrrolidinoxyl series, the reduction rate was found to be higher for the β-phosphorylated compounds, and this is probably because of the electron-withdrawing effect of the dialkoxyphosphoryl group. Nevertheless, these phosphorylated compounds show the considerable interest to present a strong coupling with the phosphorus. Since the hfsc a_P is very sensitive to the pyrrolidinyl ring conformation, aminoxyls **1**–**4** could be considered as valuable tools in all the studies realised with partially immobilised aminoxyls. TOMER-Et **1** and more especially TOMER-Pr^i **2** were shown to present a reasonable resistance to the flavin reduction, although they have been found to be more easily reduced than Proxyl or PCA, and these two β-phosphorylated probes could thus present interesting *in vivo* applications. In order to verify this hypothesis and to continue this study, further kinetic experiments are now in progress in our laboratory to evaluate the reduction rate of aminoxyls **1**–**7** in blood and in the presence of different blood constituents.

Experimental

Compounds **1**–**4** were synthesised and purified in our laboratory as previously described.[9,10] Proxyl **5** was prepared according to the method of Keana.[18] Aminoxyls **6** and **7** have been purchased from Aldrich Chemicals and used without further purification. Riboflavin, FMN, DTPA and potassium ferricyanide were provided by Sigma Chemical Company. All the buffers were stirred for 6 h in the presence of a chelating iminodiacetic acid resin, provided by Sigma Chemical Company, in order to remove trace metal impurities, and the solutions were prepared just before use in 0.4 mmol dm^{-3} phosphate buffer at pH 7.4.

EPR measurements were carried out at 20 °C in an EPR capillary tube by using a computer controlled Varian E-9 EPR spectrometer, operating at X-band with 100 kHz modulation frequency. The instrument settings were as follows: non-saturating microwave power, 10 mW; modulation amplitude, 0.2 mT; receiver gain ranging from 12 500 to 32 000; scan time, 1800 s; time constant, 1 s.

A standard sample contained 0.1 mmol dm^{-3} aminoxyl, 1 mmol dm^{-3} oxidised flavin and 1 mmol dm^{-3} DTPA. The mixture was transferred to an EPR capillary tube and argon was bubbled through the solution for at least 10 min in order to remove molecular oxygen before closing the tube. The reaction mixture was then irradiated directly in the spectrometer cavity using a slide projector lamp as the visible light source. The decrease in the low field peak of the aminoxyl was followed and the light was shut off after a loss of *ca*. half of the signal intensity. In these conditions, the concentration in photoreduced flavin was always found to be lower than 0.5×10^{-4} mol dm^{-3}. Then the aminoxyl decay was followed in the dark by recording the EPR signal at the fixed field corresponding to the first low field peak. A complete spectrum was also systematically recorded at the end of the kinetic study in order to verify the stability of the magnetic field.

Computer modelling of the kinetic curves was achieved using the program DAPHNIS elaborated in our laboratory.[10,15] Using this program, the signal at time t_n was calculated from the signal amplitude at time t_{n-1} and using a rate equation, such as eqn. (1) or (2). The standard least-squares method was then applied to fit the experimental curves, leading to the kinetic parameters and the errors in these values indicated in Tables 2 and 3.

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Paper 7/02506H
Received 11*th April* 1997
Accepted 11*th September* 1997