Comparative Study of the Reduction Rates of Various Types of Imidazoline Radicals in Tissues

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During recent years aminoxyls* have been studied as perspective contrast agents for NMR imaging in biological systems. We report a study of 30 low-molecular-weight imidazoline radicals in which we have compared the initial rates of reduction of these compounds by rat liver microsomes and ascorbate. The nuclear magnetic relaxation rate of water protons in microsomes was determined in the presence of a number of aminoxyls and it was found that the rates depend on the substituents. In microsomes the weakest oxidants among these aminoxyls are imidazolines. This type of radical is also the most resistant reduction by ascorbate.

Stable aminoxyl* radicals, that increase the relaxation rates of solvent protons, have potential utility as contrast-enhancing agents in nuclear magnetic resonance (NMR) imaging.[1-3] However, aminoxyls are reduced in tissues to non-paramagnetic species with consequent loss of their contrast properties.[4-6] The rate of aminoxyl reduction appears to be a function of oxygen concentration, the type of aminoxyl and type of cell.[7] Aminoxyl compounds with high resistance to *in vivo* reduction would, therefore, be advantageous for contrast enhancement. A systematic study of the influence of aminoxyl structure on their susceptibility to reduction has been reported by a number of authors.[4,8,9] It has been shown that spin labels that differ in ring system and chemical structure influence the rate of biological reduction. It has also been observed that pyrrolidine aminoxyls can be of greater potential value for MRI contrast enhancement than piperidine derivatives because of their greater resistance to reduction.[10]

In this paper we compare the reduction rates of various types of imidazoline radical in tissues.

Experimental

Materials.—Aminoxyls were synthesised according to the technique described by Volodarsky.[11] The manganese–radical complex was prepared by literature method[12] and was kindly provided by Ovcharenko (ITC).

Preparation of Microscomes.—Livers were obtained from male Wistar rats (120–140 g). Microsomes were prepared by the standard procedure,[13] and the protein content was determined by Lowry's method[14] using bovine serum albumin (BSA) as a standard.

Reduction of Aminoxyls by Rat Liver Microsomes.—The procedure was carried out according to a literature method.[8] To 0.02 cm^3 of microsomal suspension (usually containing 20–25 mg protein per cubic centimetre) was added the aminoxyl (0.5 mmol dm^{-3}, final concentration) and sufficient buffer (1.15% KCl containing 50 mmol dm^{-3} sodium phosphate, pH 7.4) to bring the final volume to 0.5 cm^3.

The reaction was initiated by the addition of NADPH (250 μmol dm^{-3} final concentration). The mixture was transferred to a capillary *Einmal-Mikropipetten* to measure the rate of reduction.

* IUPAC-recommended name for the commonly used nitroxide.

Reduction of Aminoxyls by Ascorbate.—Reduction was conducted in 0.1 mol dm^{-3} sodium phosphate buffer, pH 7.4, which was also 0.25 mol dm^{-3} in sucrose and 1.0 mmol dm^{-3} in EDTA. To 0.5 cm^3 of buffer solution was added a solution (5 μl) of aminoxyl in H_2O–DMSO 1:1 (v/v) (0.5 mmol dm^{-3} final concentration) followed by a solution of ascorbate (5×10^{-2} mol dm^{-3} initial concentration). The mixture was immediately transferred into capillary *Einmal-Mikropipetten* to measure the rate of reduction. Measurements were made at room temperature (22–23 °C) with a Bruker (ER–200D–SRC) electron paramagnetic resonance (EPR) spectrometer. The rate of aminoxyl reduction was determined by the decrease in the height of the low-field peak of the first-derivative spectrum (Fig. 1). This peak was chosen because the ascorbate radical anion interferes with measurements near the central aminoxyl peak.[15] In some cases, the EPR signal disappeared by the time the scan was initiated. Such aminoxyls are described as having a 'fast' rate of reduction.

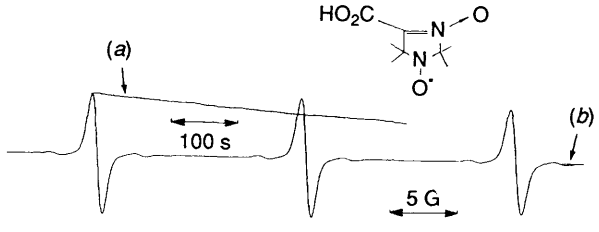


Fig. 1 Reduction of an aminoxyl by ascorbate. The sample contained 5 mmol dm^{-3} ascorbate and 0.5 mmol dm^{-3} aminoxyl (final concentrations). (*a*) time course of the magnitude of the low-field peak; (*b*) EPR spectrum at the beginning of the reduction. The spectrometer settings were microwave power 12.5 mW, gain 8×10^3, modulation amplitude 0.5 G.

Relaxivity Measurements.—A Bruker R28 (12 MHz) MR Imager was used for determining T_2.[16] Measurements were performed at 25 °C on a DMSO–H_2O solution of the radical. The standard Carr–Purcell–Meiboom–Gill sequence of 64 echoes was used to record T_2 decay.[17] Values of integrals were obtained *vs.* the corresponding time (the inter-echo delay was 56 ms) and were used to fit the non-linear function $I = A \exp(-kt/T_2)$ where I is the integral intensity for selecting a thin slice through the sample, t is the time between echoes and k is the number of echoes. A three-parameter fit was used to define T_2 by use of standard software.

Table 1 Reduction Rates of aminoxyls

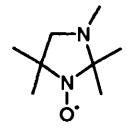
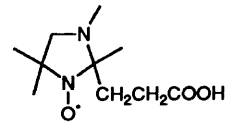
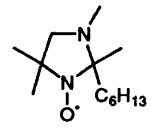
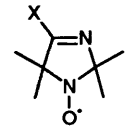
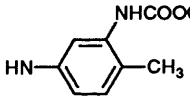
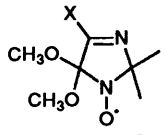
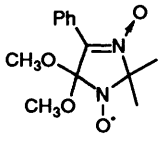
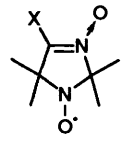
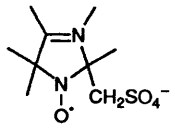
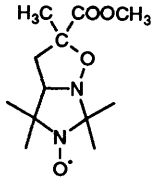
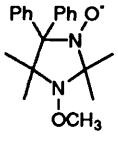
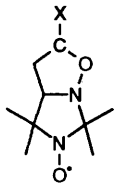
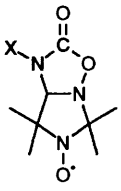
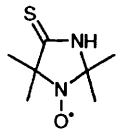
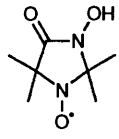
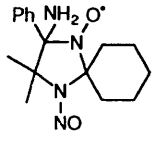
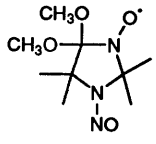
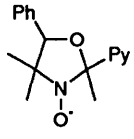
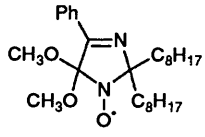
	Structure	X	Rat liver microsomes[a]	Ascorbate[b]
1			0.57 ± 0.023	3.04
2			0.41 ± 0.01	2.38
3			0.48 ± 0.02	1.33
4		NH–Ph	2.27 ± 0.17	8.99
		Ph	1.44 ± 0.04	11.1
		COONa	0.76 ± 0.07	9.81
		NH_2	1.22 ± 0.07	19.1
		$NH(CH_2)_2Cl$	1.32 ± 0.09	26.05
			2.21 ± 0.17	1.04
5		CH_3	3.95 ± 0.24	Fast
		Ph	8.95 ± 0.48	Fast
		COONa	2.42 ± 0.1	Fast
6			8.89 ± 0.19	Fast
7		NH_2	0.48 ± 0.023	Fast
		Ph	1.93 ± 0.06	Fast
		COOH	2.89 + 0.03	Fast
8			Fast	Fast
9			0.63 ± 0.023	11.23
10			30.1 ± 1.36	4.4

Table 1 *continued*

	Structure	X	Rat liver microsomes[a]	Ascorbate[b]
11		Ph	1.42 ± 0.15	Fast
		Py	1.47 ± 0.19	Fast
12		Ph	3.6 ± 0.26	Fast
		CH_2CH_2Cl	1.33 ± 0.02	Fast
13			1.2 ± 0.3	Fast
14			0.89 ± 0.11	Fast
15			Fast	Fast
16			0.82 ± 0.13	Fast
17			9.94	Fast
18			1.57 ± 0.14	Fast

[a] Initial reduction rate in nmol min^{-1} mg^{-1} protein. [b] Initial reduction rate in μmol mm^{-1} mmol ascorbate. Each rate is the average of at least three different measurements with a relative error of 15–20%.

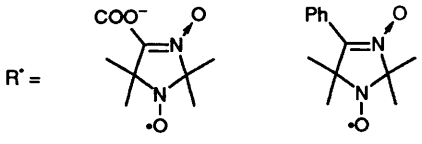
Results and Discussion

It has been previously found out that five-membered ring aminoxyls were reduced more slowly than were the six-membered derivatives. This trend was observed for both the substituted and unsubstituted heterocycles. The initial reduction rates for five-membered ring imidazoline aminoxyls are listed in Table 1. The comparison of the rates of reduction of these compounds in different systems led us to the conclusion that the

weakest oxidants among aminoxyls investigated are imidazolidine and 3-imidazoline.

For heterocyclic aminoxyls the rate of reduction of the aminoxyl group largely depends on the substituents present. For example, in cases where one of the substituents in an imine-oxide, the oxidising ability of aminoxyl group increases, owing to the strong electron-accepting effect of >C=N→O and >C=N+ groups which increases the electron deficiency on the aminoxyl group. In the case of reduction of 3-imidazoline 3-oxide aminoxyls the rate of reduction is significantly increased in the presence of substituents in the 2 and 5 positions. It was observed oxidative activity was in the order:

We also considered the data of concerning structural effects on the nuclear magnetic relaxation rate. The nuclear magnetic relaxation rate of water protons in microsomes was determined in the presence of some aminoxyls from types I (imidazoline, 0.3 ± 0.05 dm^3 mol^{-1} s^{-1}), II, 3-imidazoline (0.49 ± 0.07 dm^3 mol^{-1} s^{-1}) and III, 3-imidazoline 3-oxide (0.41 ± 0.05 dm^3 mol^{-1} s^{-1}) at 12 MHz. Note that the relaxivity of aminoxyls can be increased by the formation of a complex of the radical with a paramagnetic ion.[18] For this purpose the complexes between aminoxyl radicals and Mn ($R^{\bullet}$–Mn–$R^{\bullet}$·$2H_2O$) was obtained.



Indeed, it has been found that the T_2 relaxivities of one of the complexes determined in liver tissue, brain and blood of Wistar rats at 12 MHz are in the range 15–40 dm^3 mol^{-1} s^{-1} and about hundred times higher than that of the radical. The reduction rate of this complex (1) is 0.8 nmol min^{-1} mg^{-1} compared with 2.8 nmol min^{-1} mg^{-1} for the free radical in the microsomal system. Unfortunately it was found that these complexes dissociate in solution and cannot be recommended as contrast agents for *in vivo* experiments.

We are currently undertaking further studies to increase the stability of these paramagnetic ion–aminoxyl complexes with improved chelating properties.

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