bias-free solute–solvent effect data, which will be useful also for new solution chemistry studies (Discussion, subsection V).

Thus, our new bulbed capillary method will be established as the most reasonable, accurate, and practical referencing technique for NMR contributing also to chemistry.

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LITERATURE CITED

(1) Gordon, J. E. *J. Phys. Chem.* **1962**, *66*, 1150–1158.
(2) Creekmore, R. W.; Reilley, C. N. *Anal. Chem.* **1970**, *42*, 570–575.
(3) Weiner, P. H.; Howery, D. G. *Can. J. Chem.* **1971**, *49*, 2913–2916.
(4) Walton, H. F. *Anal. Chem.* **1972**, *44*, 256R.
(5) Zimmerman, J. R.; Foster, M. R. *J. Phys. Chem.* **1957**, *61*, 282–289.
(6) Pople, J. A.; Schneider, W. G.; Bernstein, H. J. *High Resolution Nuclear Magnetic Resonance*; McGraw-Hill: New York, 1959; pp 80–81.
(7) Tiers, G. V. D. *J. Phys. Chem.* **1958**, *62*, 1151–1152.
(8) Becker, E. D. *High Resolution NMR*, Theory *and* Chemical *Applications*, 2nd ed.; Academic Press: New York, 1980; pp 38–39, 50–51. In the Japanese translation by: Saito, H.; Shinto, H. Tokyo Kagaku Dojin: Tokyo, 1983.
(9) Landolt-Börnstein, *Sechste Auflage*, 10 teil; Springer-Verlag: Berlin, 1967.
(10) *International Critical Tables*; McGraw-Hill: New York, 1928.
(11) *Tables of the API Research Project 44*; Carnegie Press: Pittsburgh, 1953.
(12) Bothner-by, A. A.; Glick, R. E. *J. Chem. Phys.* **1957**, *26*, 1647–1650.
(13) Bothner-by, A. A.; Naar-Colin, C. *J. Am. Chem. Soc.* **1958**, *80*, 1728–1733.
(14) Reilly, C. A.; McConnell, H. M.; Meisenheimer, R. G. *Phys. Rev.* **1955**, *98*, 264.
(15) Deutsch, J. L.; Lawson, A. C.; Poling, S. M. *Anal. Chem.* **1968**, *40*, 839–840.
(16) Spanier, R. F.; Vladimiroff, T.; Malinowski, E. R. *J. Chem. Phys.*, **1966**, *45*, 4355–4356.
(17) Malinowski, E. R.; Weiner, P. H. *J. Am. Chem. Soc.* **1970**, *92*, 4193–4197.
(18) Weiss, A.; Witte, H. *Magnetochemie-Grundlagen und Anwendung*; Verlag Chemie GmbH: Weinheim/Bergstr, 1973; pp 41–45. In the Japanese translation by: Sorai, M. Misuzu Shoten: Yokyo, 1980.
(19) Bozorth, R. M. *Ferromagnetism*; Van Nostrand: New York, 1951; pp 845–849.
(20) Feher, G.; Knight, W. D. *Rev. Sci. Instrum.* **1955**, *26*, 293–294.
(21) Frei, K.; Bernstein, H. J. *J. Chem. Phys.* **1962**, *37*, 1891–1892.
(22) Mulay, L. M.; Haverbusch, M. *Rev. Sci. Instrum.* **1964**, *35*, 756–757.
(23) Rummens, F. H. A. *van der Waals forces in NMR intermolecular shielding effects* (*NMR Basic Principles and Progress*); Springer-Verlag: Berlin, 1975; Vol. 10, Chapter 15.
(24) Frost, D. J.; Hall, G. E. *Mol. Phys.* **1966**, *10*, 191–200.
(25) Momoki, K.; Fukazawa, Y. Unpublished work.

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Titration of Nitroxide Free Radicals by Nuclear Magnetic Relaxometry

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An alternative to electron spin resonance spectroscopy is proposed for the quantitative analysis of nitroxide free radicals in solution. The method is based on the chemical reduction of the paramagnetic compounds followed by NMR measurements of the longitudinal relaxation rate of the solvent protons. This titration of the nitroxides has been carried out in ethanolic solutions by reaction with known amounts of phenylhydrazine. The paramagnetic fraction of the solvent relaxation rate is precisely related to the concentration of the free radical which can be measured without prior knowledge of its specific influence on the protons relaxation rate (relaxivity). Oxygen has to be eliminated from the solutions in order to avoid reoxidation of the hydroxylamine formed. The precision of the method, tested on 11 diversely subsituted derivatives of piperidine-1-oxyl, pyrrolidine-1-oxyl, and 3-oxazolidine-1-oxyl, offers a precision of about 3%.

INTRODUCTION

Nitroxide free radicals have received a great deal of attention in biomedical magnetic resonance because of their potential uses as contrast agents for imaging (*1–3*) and as probes for pharmacokinetics (*4*) or cellular redox state (*5–8*). A knowledge of the absolute concentration of these compounds is often required, as for instance when their efficiency (or "relaxivity") as magnetic resonance imaging contrast agents is to be assessed. The calculation of relaxivity requires a determination of the concentration of the paramagnetic compounds in solution. When isolation and weighing of the pure compound are not achievable, the quantitation of it in solution is usually performed by double integration of the first derivative ESR spectrum. This kind of quantitation requires the production of standard solutions of known concentration and has a precision of about 5%. Other methods have been proposed such as titration of the iodine formed by reaction of the nitroxides with potassium iodide (*9*) or measurement of the volume of nitrogen produced when they react with phenylhydrazine (eq 1) (*10*). In the course of this chemical

$$2\text{N–O}^{\bullet} + C_6H_5NHNH_2 \rightarrow 2\text{N–OH} + C_6H_6 + N_2 \qquad (1)$$

reduction, the paramagnetic center is transformed into a diamagnetic *N*-hydroxylamine functional group (*10, 11*). A new analytical procedure based on this quenching of the paramagnetism can thus be proposed. Provided that the reaction is complete and of known stoichiometry, a true "relaxometric titration" can easily be achieved by monitoring the proton relaxation rate of the solvent as a function of the amount of phenylhydrazine added.

EXPERIMENTAL SECTION

Reagents. The Tempo (I), Tempamine (II), Tempol (III), and methyl-7-doxylstearate nitroxides (X) (Table I) are commercially available (Janssens, Beerse, Belgium and Aldrich, Brussels, Belgium). The 4-carboxytempo (IV) and the doxyl (XI) were synthesized by the procedures described by Rauckman et al. (*12*) and Keana et al. (*13*), respectively. 4-Ethoxytempo (V) was

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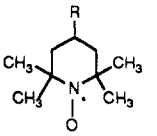
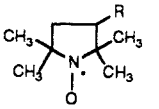
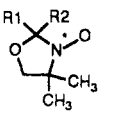
Table I. Structure of Nitroxides Studied

acronym	chemical name	no.	R group	basic structure
Tempo	2,2,6,6-tetramethylpiperidine-1-oxyl	I	-H	
Tempamine	4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl	II	$-NH_2$	
Tempol	4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl	III	-OH	
4-carboxytempo (TCA)	4-carboxyl-2,2,6,6-tetramethylpiperidine-1-oxyl	IV	-COOH	
4-ethoxytempo	4-ethoxy-2,2,6,6-tetramethylpiperidine-1-oxyl	V	$-OCH_2CH_3$	
4-propionamidotempo	4-propionamido-2,2,6,6-tetramethylpiperidine-1-oxyl	VI	$-NHCOCH_2CH_3$	
4-stearamidotempo	4-stearamido-2,2,6,6-tetramethylpiperidine-1-oxyl	VII	$-NHCO(CH_2)_{16}CH_3$	
PCA	3-carboxyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl	VIII	-COOH	
NAT	3-carboxyl(1,2,4-trihydroxybutyl-3-yl)-amide-2,2,5,5-tetramethyl-pyrrolidine-1-oxyl	IX	$-CONHCH(CH_2OH)CH(OH)CH_2OH$	
methyl-7-doxylstearate	2-(5-methoxy-5-oxopentyl)-2-undecyl-4,4-dimethyl-3-oxazolidine-1-oxyl	X	R1 = $-(CH_2)_{10}CH_3$ R2 = $-(CH_2)_5COOCH_3$	
doxyl	2,2,4,4-tetramethyl-3-oxazolidine-1-oxyl	XI	R1 = $-CH_3$ R2 = $-CH_3$	

obtained by the procedure of Maeda et al. (*14*). The amido derivatives VI and VII were prepared by reaction of Tempamine with the activated carboxylic acid derivatives prepared by reaction of dicyclohexylcarbodiimide (*15*). PCA (VIII) and NAT (IX) were generous gifts from Schering AG (Berlin). Phenylhydrazine (Aldrich) was distilled under reduced pressure before use.

Relaxometric Titration. (*a*) *Sample Preparation.* Ethanol was chosen as the reaction solvent since it dissolves all the compounds used in this work, even the highly lipophilic ones. When the samples were prepared, special care was taken to eliminate the presence of oxygen, which could reoxidize the *N*-hydroxylamine formed; solvent and samples were handled and stored under nitrogen. For each compound, a series of 11 samples were prepared by mixing 0.4 mL of an ethanolic solution of the nitroxide (concentration in the order of 0.020 M) and various aliquots (0–20 μL) of an ethanolic phenylhydrazine solution (concentration of about 0.4 M) measured with a Hamilton syringe. Samples were kept at 20 °C for 30 min. After this reaction period, nuclear magnetic longitudinal relaxation rates R_1 were measured.

(*b*) *Nuclear Relaxation Measurements.* Longitudinal relaxation rate R_1 ($R_1 = 1/T_1$) measurements were performed at 4 °C and 20 MHz on a spin analyzer Bruker Multispec PC-20 by a conventional inversion–recovery sequence. In spite of the existence of three kinds of protons in the solvent molecules, the evolution of the magnetization did not significantly differ from a monoexponential process. The values for $1/T_1$ were generally reproducible within 2%. Preliminary experiments confirmed the linear relationship between the relaxation rate of the solvent protons and nitroxide concentration in the range used for the titration. Paramagnetic relaxation rates have been corrected for the dilution due to the addition of phenylhydrazine.

Electron Spin Resonance Measurements. Electron spin resonance (ESR) spectra were recorded at room temperature and 9 GHz (0.33 T) on a Bruker 200D ESR spectrometer. The determination of nitroxide concentration was performed by double integration of the first derivative spectrum from diluted solutions (ca 10^{-4} M). The peak intensities were then compared to those of a reference solution containing weighed amounts of Tempol.

RESULTS AND DISCUSSIONS

N-Hydroxylamines produced by the reduction of nitroxides (eq 1) are diamagnetic molecules which have a negligible effect on the solvent nuclear reaction rate. Assuming that the reaction is complete, the paramagnetic relaxation rate of the solvent nuclei R_{1p} reflects the concentration of the parent paramagnetic compound through the following equation:

$$R_{1p} = R_1 - R_{1d} = r_1([\text{nit}]^\circ\ V^\circ - a[\text{red}]V_{red})/(V^\circ + V_{red})$$

where R_1 is the solvent proton relaxation rate, R_{1d} is the diamagnetic relaxation rate of the solvent free of nitroxide, r_1 is the relaxivity (s^{-1}·mmol·L^{-1}) of the nitroxide, [nit]° is the concentration of the initial nitroxide solution (mmol·L^{-1}), V° is the volume of the initial nitroxide solution (L), a is the stoichiometric factor of the reaction, [red] is the concentration of the phenylhydrazine solution (mmol·L^{-1}), and V_{red} is the volume of the added phenylhydrazine solution (L). After normalization of the paramagnetic relaxation rate to the initial volume V°

$$R''_{1p} = R_{1p}\frac{V^\circ + V_{red}}{V^\circ}$$

and including R°_{1p}, the relaxation rate of the initial nitroxide solution, we can write

$$R''_{1p} = R^\circ_{1p}\left(1 - \frac{a[\text{red}]\ V_{red}}{[\text{nit}]^\circ\ V^\circ}\right)$$

or

$$[\text{nit}]^\circ = \frac{1}{V^\circ}\frac{a[\text{red}]V_{red}}{1 - \frac{R''_{1p}}{R^\circ_{1p}}}$$

In this context, we have assumed the presence of only one nitroxide group per nitroxide molecule but the equations can easily be written for any polynitroxide. When R''_{1p} is plotted against the amount of reductant, a titration profile is obtained from which the stoichiometric factor of the reaction or the concentration of the nitroxide can easily be calculated. Figure 1 shows a typical example of such a graph obtained for the Tempamine.

In a first step, ESR was compared to the classical gravimetric technique: the concentration of solutions of Tempo, Tempol, 4-carboxytempo, and Tempamine, ascertained by weighing the amount of pure compound brought in solution was evaluated by ESR peak amplitude measurements (Table II). The relaxometric titration was then carried out on these solutions to control the stoichiometric factor of the chemical reduction (eq 1). Optimum reaction time (30 min) for the completion of the reaction was determined according to the time-dependence of the relaxation rates. Owing to the re-

Table II. Results of the Control Experiments

nitroxide	no.	10^2[nit]°, M		stoichiometric factor *a*
		weighing	ESR	
Tempo	I	2.72	2.70	1.99
Tempamine	II	2.63	2.68	2.00
Tempol	III	2.98	ref	1.98
4-carboxytempo	IV	1.91	1.95	2.00

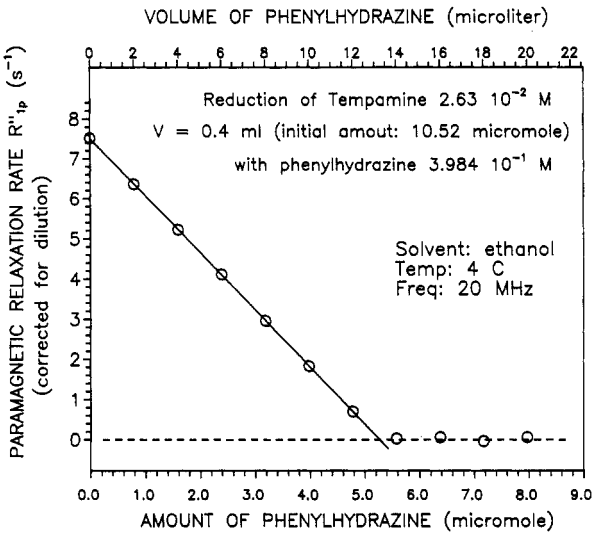


Figure 1. Relaxometric titration curve obtained for the chemical reduction of tempamine by phenylhydrazine. The dashed line represents the paramagnetic relaxation rate of the solutions free of paramagnetic compounds and is thus equal to zero. The solid line is drawn by a least-squares regression performed on the first seven data points. The parameters of the regression are $Y = 7.4955 - 1.4231X$, with a correlation coefficient of 0.9999. The intersection of the two lines allows appropriate calculations.

Table III. Comparison of Relaxometric Titration with ESR and Gravimetry

nitroxide	no.	10^2[NFR]°, M weighing	ESR	relaxometry
4-ethoxytempo	V		1.52	1.51
4-propionamidotempo	VI		1.28	1.11
4-stearamidotempo	VII		2.19	2.27
PCA	VIII	1.76	1.71	1.77
NAT	IX	1.50	1.59	1.58
methyl-7-doxylstearate	X		1.84	1.79
doxyl	XI		1.84	1.86

laxivity–precision and temperature–relaxivity relationships, measurement at low temperature is recommended. In water or alcohol, the relaxivity increase is 2-fold when the temperature is decreased from 37 to 4 °C. The quality of the results validates the procedure for quantitative analysis.

The method was then applied to the evaluation of other nitroxides solutions (Table III). Isolation of some of these compounds was not performed, but all the solutions were quantitatively assessed by ESR.

As shown in Table III, an excellent agreement between relaxometry, ESR, and, when achievable, gravimetry is attained. Other hydrazine derivatives were used as reductants but they did not offer significant advantages over phenylhydrazine which seems to be the best compromise in terms of solubility, stability, and kinetics of the reduction. It has however to be stressed that phenylhydrazine solutions must be prepared from freshly purified compound and that samples should be kept free of oxygen since reoxidation of reaction products can occur, inducing an unwanted increase in the paramagnetic relaxation rate of the solvent. This method assumes that each molecule of the nitroxide contributes the same amount to relaxation. When molecular interactions affect the relaxivity (*16*, *17*), this single value of the relaxivity is produced as a mean by fast exchange between sites. A second assumption is that the access of phenylhydrazine to the nitroxide will be the same even if the nitroxides are in two or more environments, such as partitioned between lipophilic and hydrophilic compartments. Some of these assumptions are likely to fail in solutions containing protein or in liposomal systems where reductant may also be involved in side reactions.

CONCLUSIONS

The chemical reduction of nitroxides by phenylhydrazine followed by relaxometry appears to be an efficient method for their quantitative analysis. Provided that a certain minimum of experimental precautions are taken, the precision of this relaxometric titration is around 3% and thus at least as good as the one achieved by ESR. The procedure can routinely be used as a complement or a substitute for the latter technique. Since no prior knowledge of relaxivity is required, the isolation of the pure nitroxide is not mandatory. The procedure is extremely efficient for the quantitative analysis of stock solutions in pure solvents. The extension of this protocol to polynitroxides looks straightforward. In complex mixtures like protein-containing media and liposomal systems, more complex situations due to partitioning, molecular interactions, and side reactions of the reductant should be taken into account.

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LITERATURE CITED

(1) Brasch, R. C. *Radiology* **1983**, *147*, 781–788.
(2) Brasch, R. C.; London, D. A.; Wesbey, G. E.; Tozer, T. N.; Nitecki, D. E.; Williams, R. D.; Doemeny, J.; Tuck, L. D.; Lallemend, D. P. *Radiology* **1983**, *147*, 773–779.
(3) Lurie, D.; Bussel, D. M.; Bell, L. H.; Mallard, J. R. *J. Magn. Reson.* **1988**, *76*, 366–370.
(4) Berliner, L. J.; Fuji, H. *Science* **1985**, *227*, 517.
(5) Swartz, H. M. *Advances in Magnetic Resonance Imaging*; Feig, E., Ed., Ablex Publishing Co.: Norwood, NJ, 1989.
(6) Swartz, H. M. *Acta Biochim. Biophys. Hung.* **1987**, *22*, 277.
(7) Swartz, H. M. *J. Chem. Soc., Faraday Trans. 1* **1987**, *83*, 191–202.
(8) Swartz, H. M.; Chen, K.; Pals, M.; Sentjurc, M.; Morse, P. D., II *Magn. Reson. Med.* **1986**, *3*, 169–174.
(9) Golubev, V. A.; Neiman, M. B.; Rozantsev, E. G. *Izv. Akad. Nauk SSSR, Ser. Khim.* **1966**, 343.
(10) Rozantsev, E. G.; Golubev, V. A. *Izv. Akad. Nauk SSSR, Ser. Khim.* **1966**, 891–896.
(11) Lee, T. D.; Keana, J. F. W. *J. Org. Chem.* **1975**, *40*, 3145–3147.
(12) Rauckman, E. J.; Rosen, G. M.; Abou-Donia, M. B. *J. Org. Chem.* **1976**, *41*, 564–565.
(13) Keana, J. F. W.; Keana, S. B.; Beetham, D. *J. Am. Chem. Soc.* **1967**, *89*, 3055–3056.
(14) Maeda, H.; Nakatsuji, Y.; Okahara, M. *J. Chem. Soc., Chem. Commun.* **1981**, 471–472.
(15) Bonnet, P. A.; Vallet, P.; Fernandez, J. P.; Subra, G.; Van Haverbeke, Y.; Muller, R. N.; Chapat, J. P. Unpublished work.
(16) Bennett, H. F.; Brown, R. D., III; Koenig, S. H.; Swartz, H. M. *Magn. Reson. Med.* **1987**, *4*, 93–111.
(17) Vallet, P.; Van Haverbeke, Y.; Bonnet, P. A.; Subra, G.; Fernandez, J. P.; Chapat, J. P.; Muller, R. N. *Abstract, 8th Annu Meeting, Soc. Magn. Reson. Med.* **1989**, *2*, 800.

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