PHOTOLYSIS OF CHLORPROMAZINE: HYDROXYL RADICAL DETECTION USING 2-METHYL-2-NITROSOPROPANE AS A SPIN TRAP

Y. LION, J. DECUYPER and A. VAN DE VORST

Physics Institute, University of Liège, B-4000 Liège (Belgium)

J. PIETTE

Laboratories of General and Medical Microbiology, University of Liège, B-4000 Liège (Belgium)

(Received January 11, 1982; in revised form March 16, 1982)

Summary

The spin trapping method was used to detect hydroxyl radicals during the photolysis of chlorpromazine. Two spin traps, the nitrone compound 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and the nitroso compound 2-methyl-2-nitrosopropane (MNP), were used. The minor drawbacks associated with nitrone spin traps are discussed. It was shown that a hydroxyl radical scavenger such as ethanol also inhibits the production of spin adducts initiated by the oxidation of DMPO in aqueous solution. Another strategy employing MNP and a hydroxyl radical scavenger such as dimethyl sulphoxide or alanine is described. The alkyl free radicals resulting from the reaction of the hydroxyl radicals with these scavengers are trapped by MNP. The corresponding spin adduct is more easily identified when the nitroso spin trap is used.

1. Introduction

The technique generally used to probe biological systems for evidence of free radical formation in specific processes is spin trapping associated with electron paramagnetic resonance (EPR) spectroscopy [1, 2]. This method has some advantages. The short-lived radicals react with a diamagnetic compound called the spin trap to form a long-lived radical, the spin adduct, which can often be observed at room temperature using conventional EPR equipment. In most cases the hyperfine splitting of the adduct provides information which can be used to identify the original radical. Moreover, since the stable free radicals accumulate, spin trapping is an integrative method for the quantitative analysis of radical production and is more sensitive than procedures which measure the steady state of free radicals.

Nitrone compounds, particularly 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), are most commonly used to trap oxygen-centred radicals [1]. However,

0047-2670/82/0000-0000/$02.75 © Elsevier Sequoia/Printed in Switzerland

these compounds are highly reactive and can participate in a wide variety of reactions other than radical trapping. In particular, the disadvantage of using DMPO is the possibility of oxidation of this compound into various products leading to the formation of the hydroxyl-type spin adduct DMPO–OH via a hydroxyl-independent mechanism. The precautions which must be taken when DMPO is used to detect the production of hydroxyl radicals in a biological system have recently been emphasized [3, 4]. One method of verifying that hydroxyl radical trapping has occurred is to utilize the ability of spin trapping to distinguish between different radical species. For example, it has been suggested [3] that the reaction of hydroxyl radicals with ethanol to produce α-hydroxyethyl radicals which can then react with DMPO to give an adduct exhibiting an EPR spectrum distinguishable from that of the hydroxyl adduct should be used. This technique would be very useful if the hydroxyl radical scavenger had no quenching effect on the oxidation products leading to the DMPO–OH-type adduct.

The aim of this paper is twofold: first, we investigated the effect of the most commonly used hydroxyl radical scavengers on the oxidation pathway of DMPO and secondly, taking up again the idea of employing a hydroxyl radical scavenger, we used the nitroso spin trap 2-methyl-2-nitrosopropane (MNP) to characterize the secondary radicals resulting from the reaction of hydroxyl radicals with the scavenger.

2. Materials and methods

2.1. Chemicals

DMPO and MNP were purchased from Aldrich. DMPO was purified by several adsorption cycles on activated carbon and the final concentration was measured by UV absorbance (ϵ_M = 7700 mol^{-1} l cm^{-1} at 234 nm in ethanol [3]). Chlorpromazine was obtained from Rhone-Poulenc and was used without further purification. DL-α-alanine was obtained from Fluka. All the other chemicals were reagent grade and were obtained from UCB (Brussels).

2.2. Spin trapping reactions and electron paramagnetic resonance measurements

Aqueous solutions of MNP were prepared by dissolving 10 mg of MNP in 10 ml of H_2O and stirring overnight in the dark. Dimethyl sulphoxide (DMSO) and alanine were added just prior to irradiation. Aliquots of purified aqueous solutions of DMPO were stored at —20 °C under nitrogen and were thawed just prior to use; the final concentration of DMPO was 5×10^{-2} mol l^{-1}.

Photolysis experiments were carried out *in situ* using an Osram HBO 500 mercury vapour lamp with a Schott WG 305 filter. The spectra were recorded during irradiation using a Varian E-9 spectrometer.

2.3. *UV photolysis of H_2O_2*

Concentrated H_2O_2 (30 vol.%) was freshly diluted in an aqueous solution containing DMSO or alanine and the appropriate spin trap. Irradiations were carried out for 10 s using the unfiltered output of the mercury vapour lamp described above.

3. Results

3.1. *The effect of hydroxyl radical scavengers on the oxidation of 5,5-dimethyl-1-pyrroline-N-oxide*

DMPO solutions were magnetically stirred in open test tubes in the dark for 150 min at room temperature. The formation of DMPO–OH-related EPR spectra (a_N = 14.9 G; a_H = 14.9 G) was observed during stirring.

Various solutions containing hydroxyl radical scavengers were tested. The results illustrating the growth of a DMPO–OH-type spectrum during the oxidation of DMPO are shown in Table 1. Among the different hydroxyl radical scavengers tested, it is obvious that ethanol can inhibit the formation of the DMPO–OH-type adduct. Moreover, the inhibition is accompanied by the appearance of a new signal, the splitting of which is very close to that observed for the α-hydroxylethyl adduct in the same solvent composition (50 vol.%). Furthermore, the photolysis of H_2O_2 in the same solvent mixture generated only α-hydroxylethyl adducts, the hydroxyl radical adducts being completely quenched. The other scavengers did not quench the formation of DMPO–OH-type adducts and accordingly are reliable hydroxyl radical scavengers. However, the use of ethanol as a scavenger can lead to false conclusions regarding the presence of hydroxyl radicals.

3.2. *Indirect detection of the hydroxyl radicals using 2-methyl-2-nitrosopropane as the spin trap*

The MNP spin trap does not show the drawbacks associated with DMPO which were discussed above. This spin trap is extensively used to trap the alkyl radicals produced during the radiolysis and photolysis of biomolecules in aqueous solutions [5].

TABLE 1

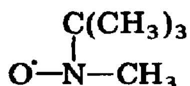
Low field intensity (corrected for solvent effects) of the DMPO–OH electron paramagnetic resonance spectrum observed in aerated solutions after magnetic stirring

Mixture	*Relative intensity*
DMPO–(H_2O)	100
DMPO–(50vol.%H_2O–50vol.%ethanol)	55
DMPO–(50vol.%H_2O–50vol.%*tert*-butanol)	122
DMPO–(50vol.%H_2O–50vol.%isopropanol)	220
DMPO–(50vol.%H_2O–50vol.%0.1 M sodium benzoate)	128

In the nitroso spin trap the scavenger radical is directly attached to the nitroxide nitrogen giving rise to a specific pattern which facilitates its identification. As it is known that hydroxyl radicals are not easily trapped by nitroso compounds, it is necessary to use a scavenger that will produce secondary radicals that will subsequently be trapped by MNP. These scavengers must also produce secondary radicals characterized by spin adducts associated with specific EPR spectra. Since MNP is photosensitive and produces di-*tert*-butyl nitroxide (DTBN) after photolysis, hydroxyl radical scavengers that will generate spin adduct spectra rich in lines that are well separated from the DTBN lines must be used.

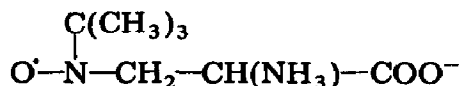
Two hydroxyl radical scavengers meeting these conditions are DMSO and DL-α-alanine. DMSO was chosen because it is a compound that is widely used to protect deoxyribonucleic acid (DNA) from hydroxyl radicals generated either in γ radiolysis experiments or by Fenton's reagent [6].

The reaction of hydroxyl radicals with DMSO liberates $^{\bullet}CH_3$ [7]; the structure of the expected corresponding spin adduct with MNP is



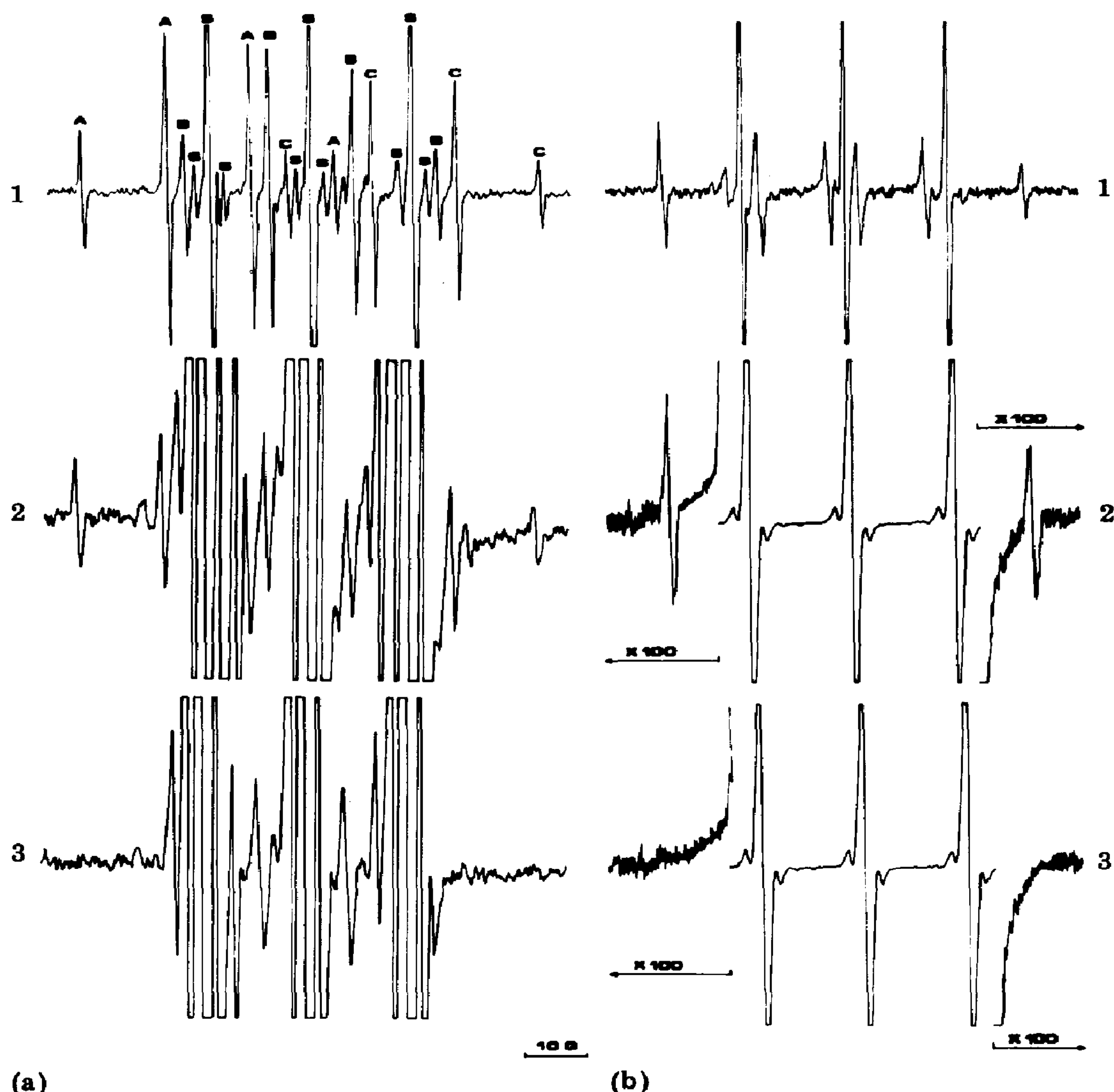
To test our method we chose a photosensitizer known to produce strand breaks in DNA but for which the production of the hydroxyl radical had not been demonstrated [8]. The irradiation of chlorpromazine with light of wavelength greater than 305 nm in the presence of MNP and DMSO gave rise to the spectrum shown in Fig. 1(a), curve 2. It consists of a primary triplet (17.20 G) further split into a quartet of relative intensity 1:3:3:1 by the interaction with three equivalent hydrogens (a_H = 14.20 G). However, most of the lines are hidden behind the DTBN spectrum (a_N = 17.20 G). In order to confirm the assignment, the CH_3 radicals were generated by photolysing an aqueous solution containing the same concentration of DMSO in the presence of H_2O_2 (Fig. 1(a), curve 1). The outer lines marked A and C correspond exactly to each other in the two spectra indicating the presence of the same radical species.

A second set of experiments was performed using DL-α-alanine as the hydroxyl radical scavenger. It is known [9] that the reaction of the hydroxyl radicals with alanine gives rise to hydrogen abstraction radicals which can be trapped by MNP, generating the spin adduct



The spectrum obtained when an aqueous solution containing chlorpromazine, DL-α-alanine and MNP was irradiated with light ($\lambda > 305$ nm) is illustrated in Fig. 1(b), curve 2.

The lines located at the edges of the spectrum are very weak; however, they are situated at the same magnetic field value as the lines observed when



(a) (b)

Fig. 1. (a) EPR spectra of DMSO–MNP solutions (curve 1, containing H_2O_2 and photolysed with UV light; curve 2, containing chlorpromazine and illuminated with light of wavelength greater than 305 nm; curve 3, illuminated with light of wavelength greater than 305 nm in the absence of chlorpromazine; the lines marked A, B and C are characteristic of the triplet of quartets associated with the CH_3 adduct and the lines labelled S are due to the DTBN radical); (b) EPR spectra of DL-α-alanine–MNP solutions (curve 1, containing H_2O_2 and photolysed with UV light; curve 2, containing chlorpromazine and illuminated with light of wavelength greater than 305 nm; curve 3, illuminated with light of wavelength greater than 305 nm in the absence of chlorpromazine).

alanine is photolysed in the presence of H_2O_2 (Fig. 1(b), curve 1) (a_N = 16.30 G; $a_H{}^{\beta}$ = 13.70 G; the splitting of the γ hydrogen is not seen because of the low resolution; the modulation amplitude was 1 G).

4. Conclusion

It has been shown that, in systems suspected of producing hydroxyl radicals, the joint use of a specific hydroxyl radical scavenger and the nitroso spin trap MNP is an unambiguous method of detecting the presence of hydroxyl radicals. Although the observed spin adduct results from a secondary reaction between MNP and the specific radical produced by the scavenger, the characteristic hyperfine structure helps to identify it as the result of the reaction of hydroxyl radicals with the scavenger. This is a great advantage compared with the six-line spectrum usually obtained with DMPO.

We are now investigating other systems in our laboratory, in particular DNA–proflavine complexes. Preliminary results indicate that hydroxyl radicals are probably present when the complex is irradiated with visible light.

Acknowledgment

J. Piette is a Senior Research Assistant supported by the National Fund for Scientific Research, Belgium.

References

1 E. G. Janzen, in W. A. Pryor (ed.), *Free Radicals in Biology*, Vol. 4, 1980, p. 115.
2 M. J. Perkins, *Adv. Phys. Org. Chem.*, *17* (1980) 1.
3 E. Finkelstein, G. M. Rosen and E. J. Rackman, *Arch. Biochem. Biophys.*, *200* (1980) 1.
4 H. Nohl, W. Jordan and D. Hegner, *FEBS Lett.*, *123* (1981) 241.
5 P. Riesz and S. Rustgi, *Radiat. Phys. Chem.*, *13* (1979) 21.
6 J. E. Repine, O. W. Pfenninger, D. W. Talmage, E. M. Berger and D. E. Pettijohn, *Proc. Natl. Acad. Sci. U.S.A.*, *78* (1981) 1001.
7 W. T. Dixon, R. O. C. Norman and A. J. Buley, *J. Chem. Soc.*, (1964) 3625.
8 H. Fujita, A. Endo and K. Susuki, *Photochem. Photobiol.*, *33* (1981) 215.
9 S. Rustgi, A. Joshi, H. Moss and P. Riesz, *Int. J. Radiat. Biol.*, *31* (1977) 415.