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Ryszard Krzyminiewski[1], Zdzislaw Kruczynski[1], Aleksander Stepien[2], Bernadeta Dobosz[1]

Free radicals in a conglomerate of peripheral blood with a spin trap investigated by the EPR method before and after angioplasty treatment

[1]Adam Mickiewicz University, Faculty of Physics, Medical Physics Division,
Umultowska 85, 61-614 Poznan, Poland
[2]Regional Hospital in Kielce, Poland
e-mail: benia@hoth.amu.edu.pl

The Electron Paramagnetic Resonance (EPR) was used to investigate free radicals in the peripheral blood of patients subjected to angioplasty treatment. To detect these free radicals, a nitrosobenzene spin trap was used in this experiment. The EPR spectra of the blood with a spin trap conglomerate was measured at room temperature and at 170 K. To confirm the kind of free radicals in the conglomerate blood-spin trap, simulation and quantum-chemical calculations were made, and the conglomerate spin trap with ascorbic acid was measured. Two different types of radicals, one at room temperature and the other in a frozen sample of blood, were found.

Key words: Electron Paramagnetic Resonance, Nitrosobenzene, radicals.

Introduction

The production and reactions of free radicals in biological systems and also in the human body is a very important problem. The most important oxygen free radicals (OFRs), which are constantly formed in the human body by metabolic processes, are discussed in literature [1, 7, 9-10]. Reactive oxygen radicals such as superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and others have become increasingly implicated in human diseases [10]. In human cells, superoxide is quickly transformed

into hydrogen peroxide (H_2O_2). This reaction is greatly accelerated by superoxide dismutase (SOD), a widely distributed enzyme. H_2O_2 in sufficient concentrations will kill any cell. Further reduction in H_2O_2 labilizes the interoxygen bond, resulting in a cleavage to produce OH^- and $^{\bullet}OH$. The hydroxyl radical is a highly reactive radical species.

Cardiovascular disease is a heterogeneous group of disorders that affects the heart and blood vessels. These diseases are characterized by angina pectoris, hypertension, congestive heart failure, acute myocardial infarction (heart attacks), stroke, and arrhythmia. There is a connection between free radical reactions, inflammatory states and cardiovascular diseases [9-10].

Reperfusion of the ischemic myocardium during angioplasty can restore oxygen and substrates to the ischemic myocardial cells, but this process may create another form of myocardial damage, termed 'reperfusion injury' [3-4, 6, 8, 16-17]. Thus, restoration of a normal blood flow in the heart by methods such as angioplasty, thrombolytic agents or cardiopulmonary bypass can lead to specific lesions (arrhythmias, deficit in contractility, necrosis), the importance of which also depends on the duration of ischemia.

The influence of free radicals is discussed in a large number of papers, but there are only few methods which make it possible to measure free radicals in the blood. One of the direct measurement methods is Electron Spin Resonance (ESR) and spin trapping methodology [5-6, 15].

Furthermore, it is currently believed, based on experimental findings, that in the ischemic tissue some impairment occurs in antioxidant mechanisms. Evidence to support this statement comes also from the cardioprotective effects of agents capable of inducing antioxidant enzymes in the heart and from the beneficial effects of several enzymatic free radical scavengers, antioxidants and iron chelators in a reperfused myocardium [14].

The proposed concept of the role of free radicals in reperfusion injury has important implications in cardiac surgery and heart transplantation.

The aim of this study was to find what kind of free radicals are generated in a complex of human blood and spin trap after angioplasty treatment and to determine the most appropriate experimental conditions such as the temperature of measurements and the concentration of spin trap-blood solution, for determining free radicals parameters.

Material and Methods

Sixty people participated in the treatment between March 2006 and December 2007. They were hospitalised because of acute coronal syndrome and were treated by primary coronal angioplasty in the Haemodynamics Laboratory at Swietokrzyskie Cardiology Centre of the Provincial Joint Hospital in Kielce, Poland.

The blood from the peripheral vein was taken directly before and directly after angioplasty to determine biochemical and inflammatory parameters. A resting cardiogram was made and then twenty-four hours after angioplasty, another sample of blood was taken and an ECG examination was made again. The last examination was carried out six months after hospital treatment.

A sample of 0.2 ml of the patient's blood was mixed with 4 ml of nitrosobenzene solution immediately after blood samples' collection and divided into three identical samples. After one minute, these samples were placed in the Dewar flask with liquid nitrogen (77 K) and stored.

The nitrosobenzene spin trap C_6H_5NO obtained from Aldrich Chemical Co. was used in the experiments. 100 mg of nitrosobenzene was dissolved in 3 ml of methanol CH_3OH, and 97 ml of distilled water was added.

The ascorbic acid, obtained from Sigma, also used in the experiment, was dissolved in the following proportions: 176 mg of acid in 100 ml of distilled water.

The measurements were made with a Bruker EMX-10 X-band (9.4 GHz) spectrometer with magnetic field second modulation frequency of 100 kHz. The EPR spectra were recorded at room and 170 K temperatures and in a sweep width of 20 mT. Low temperatures were maintained by a Bruker temperature controller ER 4131VT. The following time parameters were applied: a sweep time of 10.486 s and a time constant of 20.480 ms.

The EPR spectra were simulated in WINEPR SimFonia program from Bruker.

Quantum-chemical calculations were made using a Gaussian98 program. Geometric structures were optimized with the B3LYP/6-31G**, and the A-tensor was calculated by the B3LYP/EPR-II method also implemented in Gaussian98.

Results and discussion

In the first part of the experiment, temperature measurements were carried out to choose appropriate conditions for EPR measurements and free radicals identification. The temperature of 170 K was used as the optimum value for measurements. At this temperature, the concentration of radicals can be measured most easily and with the highest accuracy. It was also found that the best conditions for sample storage with stable concentration of trapped radicals are provided by the temperature of liquid nitrogen (77 K).

For the patients undergoing treatment, all their EPR spectra were similar but varied in intensity. On the repeat of the measurements and estimation of errors in free radical concentration, every time three samples of the blood with a spin trap in the same patient were measured. The error in free radical concentration measurements in three control samples for each patient was estimated at about 10%.

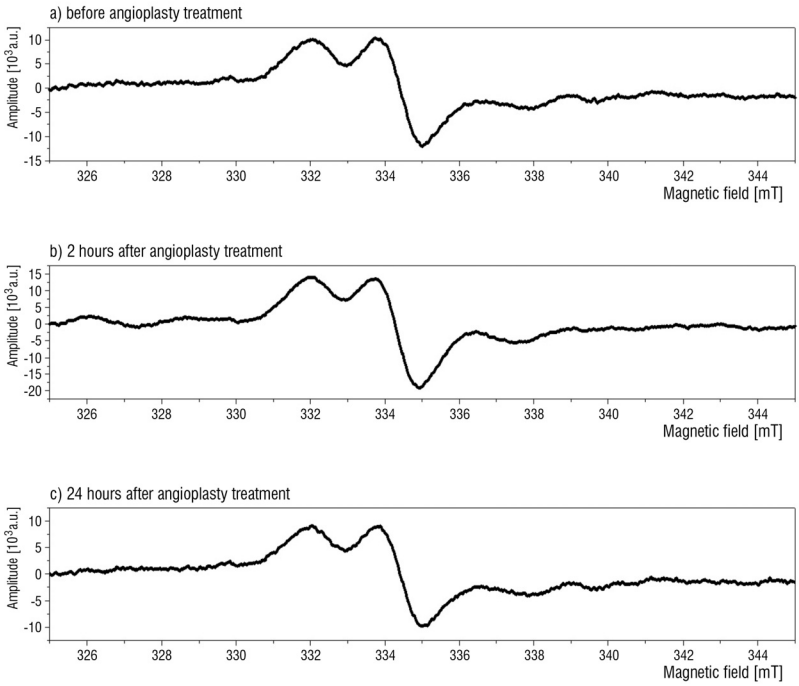

Figure 1. Typical EPR spectra of trapped free radicals in a patient's blood recorded at 170 K: (a) before angioplasty, (b) 2 hours after angioplasty, and (c) 24 hours after angioplasty treatment

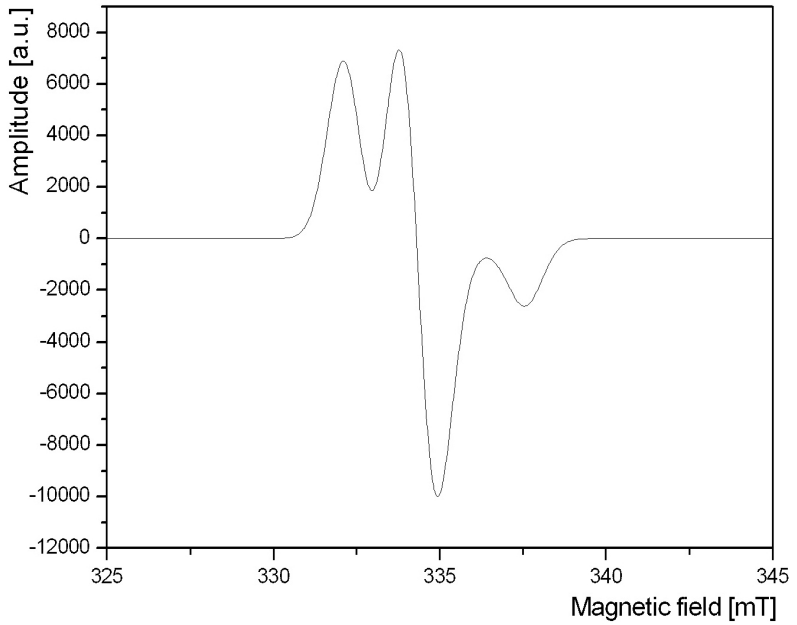

Figure 2. Simulated spectrum for parameters g1 = 2.01, g2 = 2.0068, g3 = 2.0032, A1 = 0.25 mT, A2 = 0.25 A3 = 2.68 mT, width1 = 1.0 mT, width2 = 1.0 mT, width3 = 1.0 mT

A typical EPR spectra of trapped radicals in human blood recorded at 170 K are shown in Figure 1. Figures 1a, b and c show the spectra before, 2 hours after and 24 hours after angioplasty, respectively. In these spectra, the shape of the EPR signals is similar, but they differ as to their free radical concentrations. The biggest concentration of free radicals is observed 2 hours after angioplasty and it decreases 24 hours after this treatment. These results are in good agreement with those obtained by Zweier [16-17]. He and his team observed a burst of free radical generation during the early seconds of post ischemic reperfusion in the frozen tissue of rabbit hearts.

The simulated EPR spectrum, which we obtained using the WINEPR SimFonia program from Bruker, is shown in Figure 2. The following spectroscopic parameters were used: g1 = 2.01, g2 = 2.0068, g3 = 2.0032; A1 = 0.25 mT, A2 = 0.25 mT, A3 = 2.68 mT; with the widths for tensor A: width1 = 1.0 mT, width2 = 1.0 mT, width3 = 1.0 mT, and they were typical for the rigid-limit radical $^{\bullet}$NO type [12-13]. There is a good agreement for these two experimental and simulated spectra (Figures 1 and 2).

The EPR spectrum of radicals trapped in human blood was also recorded at room temperature (Figure 3a). This spectrum is much more complicated than the one recorded at 170 K (Figure 1) because of fast rotation of the free radicals. Here four groups of lines there are visible, each group consisting of four triplets. The simulated EPR spectrum, shown in Figure 3b, was obtained for the following parameters:

– Isotropic factor g=2.0055;
– Hyperfine splittings in mT;
– $A_N = 1.055$; $A_H^{NO} = 1.296$; $3A_H^{o,p} = 0.338$; $2A_H^m = 0.105$.

This spectrum is very similar to that obtained by Mottley for radicals in nitrosobenzene with ascorbic acid [11].

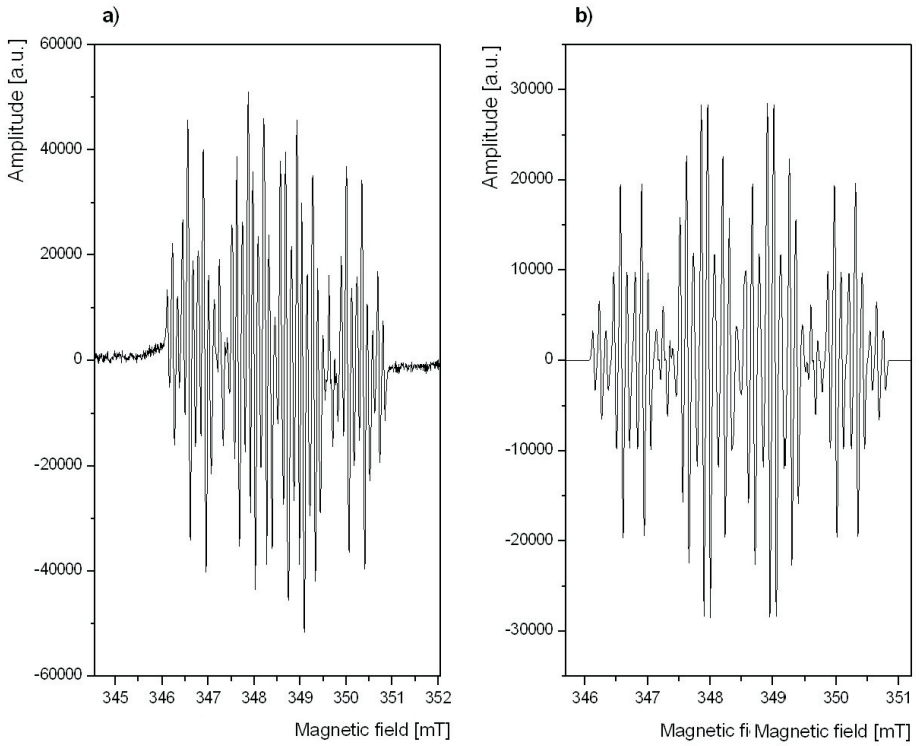

Figure 3. Typical EPR spectra of trapped free radicals in human blood: (a) experimental spectra recorded at room temperature, and (b) simulated spectra for the following parameters: isotropic factor g = 2.0055; Hyperfine splittings in mT: $A_N = 1.055$; $A_H^{NO} = 1.296$; $3A_H^{o,p} = 0.338$; $2A_H^m = 0.105$

On the basis of the results obtained in the experimental, simulated and literature data, it was claimed that radicals in the blood at room temperature are nitrosobenzene radicals with H attached to the NO group (Figure 4). The unpaired electron is delocalized on the benzene ring and the NO group (dotted line in Figure 4). The maximum densities are at N and ortho and para positions (Figure 4).

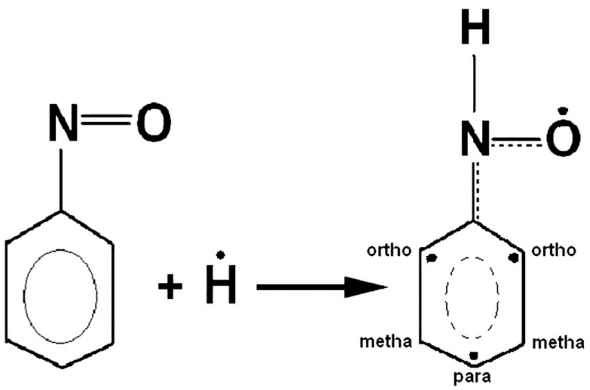

Figure 4. A scheme of nitrosobenzene radicals creation in blood

It is necessary to point out that, using the hyperfine splitting values determined above ($A_N = 1.055$; $A_H^{NO} = 1.296$; $3A_H^{o,p} = 0.338$; $2A_H^m = 0.105$) for rigid-limit EPR spectra simulation, it is not possible to obtain the spectrum shown in Figure 1. As seen from simulation (Figures 2 and 3b), the best fitting for the experiment at 170 K (Figure 1) is obtained when the hyperfine splitting constant A_H^{NO} is assumed to be very small, less than 0.5 mT. To determine the appropriate type of free radical responsible for Figure 1, the quantum-chemical calculations in the Gaussian98 program were made for three assumed radicals:

1. with H connected to N in NO group,
2. nitrosobenzene anionoradical,
3. with H connected to O in the NO group.

The results are shown in Figure 5 and in Table 1. From quantum-chemical calculations, it can easily be seen that two different kinds of free radicals must be present — one at a low temperature (170 K) and the second one at room temperature. This interpretation explains the EPR spectrum of the blood with a spin trap at room temperature with radical (1), which converts at 170 K in radical (2) (Figures 4 and 5).

Table 1. The results from quantum-chemical calculation for the assumed radicals.

Atom	Isotropic Fermi Contact Couplings [mT]		
	(NH–O) *	NO *	N–OH *
7H	0.14	0.16	0.21
8H	−0.33	−0.38	−0.48
9H	−0.3	−0.36	−0.5
10H	0.1	0.11	0.21
11H	−0.37	−0.52	−0.48
12N	1.06	1.17	1.36
13O	−1.54	−0.92	−1.04
14H	−1.29	—	−0.47

* (NH–O) — the radical formed by H attachment to N, NO — anionoradical, N–OH — the radical formed by H attachment to O

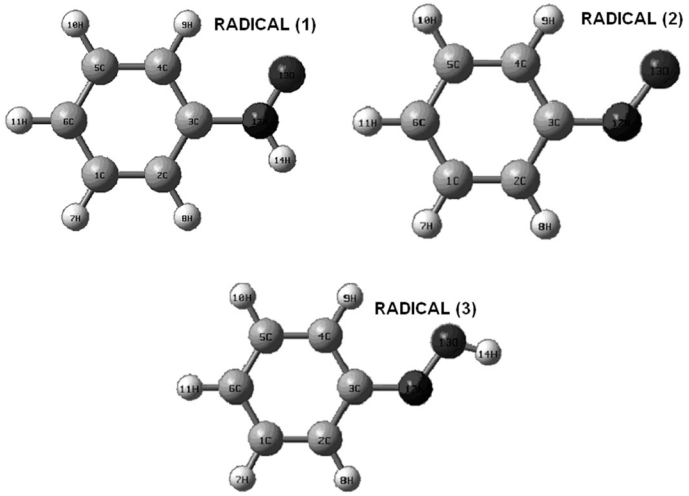

Figure 5. Free radicals assumed for quantum-chemical calculations:
(1) — with H connected to N in NO group, (2) — nitrosobenzene anionoradical, and
(3) — with H connected to O in NO group

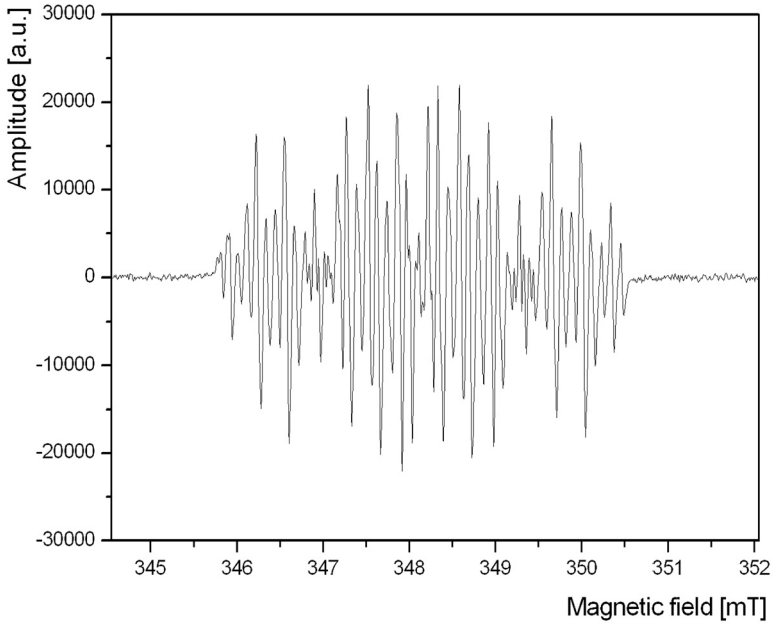

Figure 6. A typical EPR spectrum of trapped free radicals in a conglomerate of a spin trap with ascorbic acid recorded at room temperature

To verify this statement and to confirm the existence of two different types of free radicals in the blood-spin trap conglomerate, a series of measurements with ascorbic acid-spin trap conglomerate was made. From literature it is known that this kind of free radical is generated by the reduction of nitrosobenzene to a free radical by ascorbate [2, 11]. Typical spectra of such a conglomerate recorded at room temperature and 170 K are shown in Figures 6 and 7. The results of room temperature measurements confirm the same type of free radical formed by H added to the NO group (Figure 5, radical (1)) as those assumed at room temperature for the conglomerate blood-spin trap. The EPR experimental and simulated spectra of the frozen spin trap-ascorbic acid conglomerate (Figures 7a and 7b) confirm the presence of radical (1) at 170 K and they are different from the EPR spectrum of the frozen spin trap-blood conglomerate (Figure 1). In this way, the creation of a different kind of free radical in the human blood-spin trap conglomerate at room and low temperatures was confirmed. At present, nitrosobenzene anionoradical can be assigned for the blood-spin trap conglomerate at 170 K, because the isotropic hyperfine splitting A_N at 170 K has a range of 1.1 mT, and the hyperfine

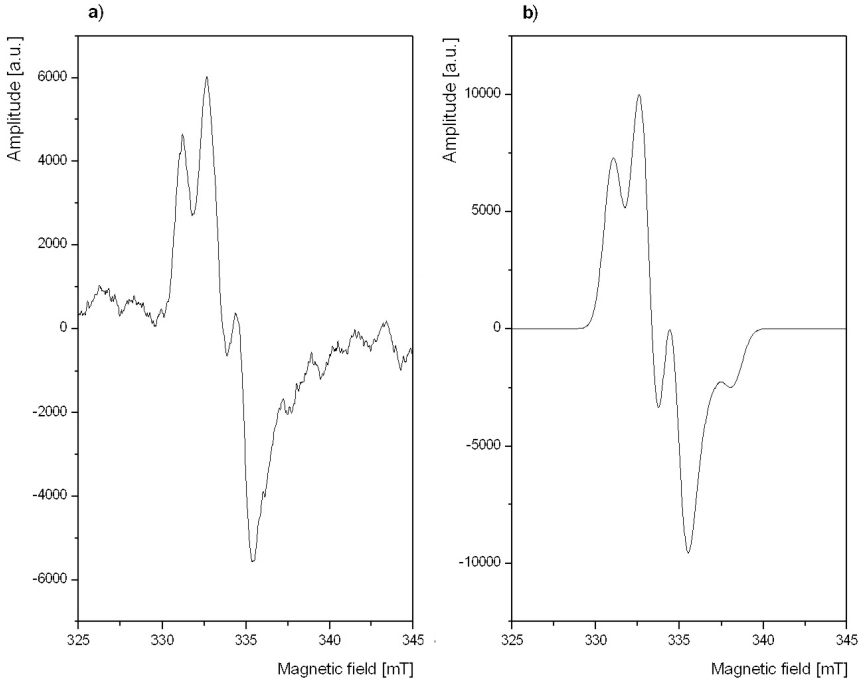

Figure 7. Typical EPR spectra of trapped free radicals in a conglomerate of a spin trap with ascorbic acid: (a) experimental spectra recorded at 170 K, and (b) simulated spectra for the following parameters: g1 = 2.01, g2 = 2.0068, g3 = 2.0022, and hyperfine splittings in mT: for nitrogen N: A1 = 0.25, A2 = 0.25 A3 = 2.68; for hydrogen attached to nitrogen: A1 = 2, A2 = 1.5, A3 = 1.5; $3A_H^{o,p} = 0.3$; $2A_H^m = 0.1$; width1 = 1.0 mT, width2 = 1.0 mT, width3 = 1.0 mT

splitting from hydrogen connected to nitrogen is absent, which is in good agreement with quantum-chemical calculations for anionoradicals (Table 1).

To check the possible conversion of free radicals, a series of temperature experiments were made. A blood sample was frozen down to 170 K, then heated to room temperature, kept at this temperature for about 15 minutes and frozen again to 170 K. Each time, an EPR spectrum was recorded. Both EPR spectra recorded at 170 K (before and after heating) were identical (Figure 1). It can be stated that conversion of a radical created from H connection to N in anionoradical is a convertible process. Probably it is caused by the creation and breaking off of intermolecular hydrogen bonds or protons moving from the $(-\mathrm{NHO})^{\bullet}$ group to protein components.

Conclusions

On the basis of EPR results, it was found that in people with an inflammatory state (after angioplasty treatment) a nitrosobenzene spin trap can be used to detect free radicals in the blood conglomerate with a spin trap.

Blood samples with a spin trap should be kept at liquid nitrogen temperature to stabilize the concentration of free radicals in time, and the best temperature to measure the concentration of trapped radicals in these samples is 170 K.

The conversion of a radical created by a hydrogen connection to nitrogen at room temperature into anionoradical at 170 K is a convertible process.

Acknowledgements

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