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Vol. 129 (2016) ACTA PHYSICA POLONICA A No. 2

Proceedings of the 50th Zakopane School of Physics, Zakopane, Poland, May 18–23, 2015

NMR Relaxation Measurements as a Tool for Observation of Oxidative Processes

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Water proton relaxation times, T_1 and T_2, were measured to assess the kinetics of the oxidative processes in biological samples. The oxidation in aqueous solutions of albumins was promoted by an addition of 3% hydrogen peroxide (H_2O_2). Immediately following this addition a sharp exponential decrease of both relaxation times was observed. As we confirmed experimentally, the time course of relaxation depended on several essential factors like structure and the concentration of proteins and also the presence of antioxidants added to solution. In experiments with protein solutions containing a small amount of ascorbic acid, after reaching a minimum, relaxation time increased towards the initial (pre-addition H_2O_2) values. We conclude that this T_1 and T_2 recovery is a consequence of the presence of antioxidants and may be used to evaluate its action. This study demonstrates that nuclear magnetic resonance (NMR) relaxation measurements may be useful in evaluating free radicals reactions and antioxidants capacity.

DOI: 10.12693/APhysPolA.129.226

PACS: 76.60.–k, 76.90.+d, 76.30.Rn

1. Introduction

A number of biological processes produce reactive oxygen species (ROS), including hydrogen peroxide. Cytotoxic action of ROS includes proteins, lipid and DNA damage and can be a primer for many diseases. Therefore investigations of ROS are important in medical research and in assessing the influence of environmental pollutants on health.

To date, among magnetic resonance methods, only electron paramagnetic resonance (EPR) [1] and chemically induced dynamic nuclear polarization (CIDNP NMR) [2] have been used to evaluate the effects of free radicals on protein solutions. However these techniques require special sample preparation (freezing and drying) which is often destructive to native protein structure.

In this work we applied a relatively straightforward and non-invasive NMR relaxation method to assess oxidation processes in biological systems.

2. Materials and methods

Dry, lyophilized, powdered egg white albumin (EWA, MW 44 287 Da, Polskie Odczynniki Chemiczne, POCH Poland) and bovine serum albumin (BSA, MW 66 382 Da, Sigma-Aldrich) were used in the study. Protein solutions at concentrations ranging from 4% to 25% (by weight) were prepared by dissolving lyophilized powder in double distilled and de-ionized water.

Crystalline ascorbic acid (vitamin C, Polskie Odczynniki Chemiczne, POCH Poland) was dissolved in water forming a saturated solution (≈33% at room temperature) and added to protein solutions in 1:5 proportion.

The 3% hydrogen peroxide solution (purchased at pharmacy) was added immediately before measurements.

Longitudinal T_1 and transverse T_2 relaxation times were measured using a Minispec Bruker spectrometer operating at 1.5 T (proton resonance frequency of 60 MHz). Measurements were performed at stabilized room temperature 23 °C ± 1 °C. Spin–lattice relaxation time T_1 was measured using an inversion recovery (IR) sequence ($\pi-\tau-\pi/2$–FID) and spin–spin relaxation time T_2 using a CPMG sequence.

Data was analyzed assuming monoexponential relaxation for T_1 and T_2 using standard Bruker procedures.

3. Results and discussion

Previously we have observed kinetics of oxidation processes initiated by the addition of hydrogen peroxide to blood serum samples [3]. Complete understanding of relaxation mechanisms in complicated biological systems like blood serum or tissue is not easy. Because NMR relaxation in serum is mostly determined by the presence of proteins and paramagnetic ions, in order to analyze the relaxation processes we need to consider both of these factors [4]. Consequently we performed experiments with simple systems like solutions of well studied diamagnetic protein (EWA (egg white) and BSA (blood serum) albumins), eliminating paramagnetic relaxation and considering protein solutions only.

Some representative examples of results of T_1 experiments obtained for protein solutions are presented in Figs. 1 and 2. Error bars are not shown as the error was less than 2% of the total value for each data point.

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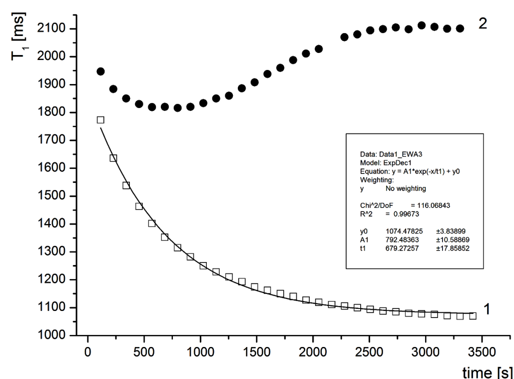

Fig. 1. The time dependence of T_1 following the addition of H_2O_2 in 1:10 proportion to 7% EWA solution (curve 1) and to 7% EWA solution containing vitamin C (curve 2).

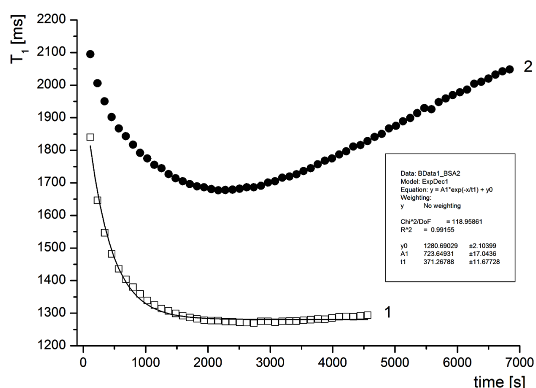

Fig. 2. As in Fig. 1, but for 6.7% BSA solution.

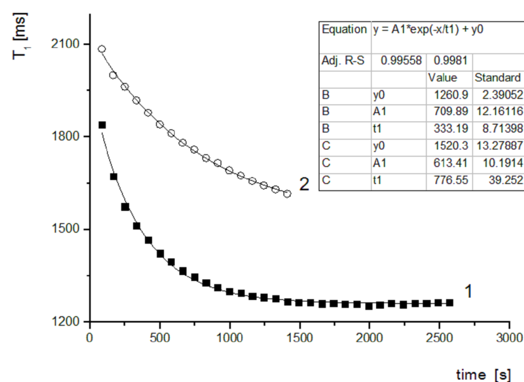

Fig. 3. The time dependence of T_1 following the addition of H_2O_2 in 1:10 proportion to 10% EWA solution (curve 1) and to 4% EWA solution (curve 2).

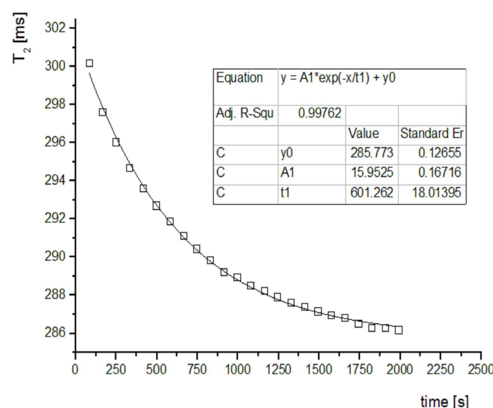

Fig. 4. The time dependence of T_2 following the addition of H_2O_2 in 1:10 proportion to 10% EWA solution.

Figure 1 shows the time dependence of T_1 following the addition of H_2O_2 in 1:10 proportion to 7% EWA solution (curve 1) and to 7% EWA solution containing vitamin C (curve 2).

Figure 2 shows the time dependence of T_1 following the addition of H_2O_2 in 1:10 proportion to 6.7% BSA solution (curve 1) and to 6.7% BSA solution containing vitamin C (curve 2).

Hydrogen peroxide is one of the strongest reactive oxygen species and when added to aqueous protein solution it causes creation of free radicals which shorten the water proton relaxation times (T_1, T_2) [5]. The relaxation times were not stable over time because of free radicals progressive damage to protein structure.

Immediately after addition of H_2O_2 to the aqueous protein solutions, both relaxation times, T_1 and T_2, decreased exponentially reaching a plateau after about 2500 s. The rate of T_1 decrease was dependent on the type of protein and its concentration. For the EWA solution the fitted line had an exponent index 679 ± 17 s (Fig. 1) and for BSA the index was 371 ± 14 s (Fig. 2). For 10% and 4% EWA solution the indexes were 333 ± 8 s and 776 ± 39 s respectively (Fig. 3).

In spite of much faster spin–spin relaxation ($T_2 < T_1$) similar time dependence of T_2 was observed in parallel experiments with the same samples (Fig. 4). However, measurements of T_1 are more useful for observation of the kinetics of oxidative processes because of their independence on H_2O_2 concentration up to high values [3].

Vitamin C (L-ascorbic acid) was chosen for demonstration of anti-oxidizing action in proteins solutions. It is a well-known powerful antioxidant which prevents the oxidation of other substances by undergoing oxidation itself. It is very soluble in water and at low concentration (less than 3%) changes the pH of solution from 7 to 6 [6]. Ex-

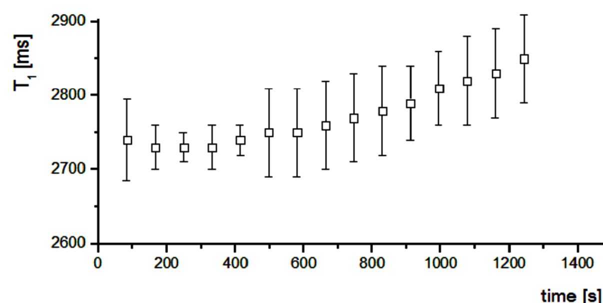

Fig. 5. The time dependence of T_1 following the addition of H_2O_2 in 1:2 proportion to saturated aqueous solution of ascorbic acid.

periments performed with saturated aqueous solution of ascorbic acid after addition of 3% H_2O_2 demonstrated no significant changes in T_1 (Fig. 5). Therefore we assume that in our experiments vitamin C addition had negligible pH effect on T_1 relaxation.

In protein solutions with the vitamin C addition, the time course of T_1 after adding H_2O_2 was different than in aqueous protein solution. After reaching the minimum, T_1 increased. The depth and position of the minimum depended on concentration of vitamin C present in solution. The minimum became shallower with increasing vitamin C concentration [3]. We conclude that this T_1 re-growth is a consequence of the anti-oxidizing action of vitamin C in solution which prevents proteins from oxidative damage.

We have observed in further experiments (not published yet) that the kinetics of the processes discussed here are dependent on the amount of H_2O_2 added, on concentration and structure of the protein, and on the presence of antioxidants.

Results of our measurements are in agreement with EPR studies of the time dependence of peroxide radical concentrations (evaluated from signal intensity) after addition of H_2O_2 to protein solution [1].

4. Conclusions

The NMR relaxation method presented here may be helpful in the investigation of the kinetics of oxidative processes in biological solutions. This method is noninvasive and could be used *in vivo*. It indicates that measurements of NMR relaxation times may be used for studies of the efficiency of antioxidants and estimation of free radical creation following ionizing radiation [5, 7]. We hope that in the future NMR relaxation measurements might also be useful in the diagnosis of some diseases, especially those involving free radical production.

Further experiments are in progress. We are currently investigating the effect of H_2O_2 on other biological systems.

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