ELSEVIER

Biochimica et Biophysica Acta 1190 (1994) 123–128

BBA Biochimica et Biophysica Acta

Free radical reactions of MTDQ and its effect on biological membrane

Joseph Belágyi *, Béla Török, László Pótó

Central Research Laboratory, University Medical School, 12 Szigeti u., H-7643 Pécs, Hungary

(Received 23 August 1993)

Abstract

The effect of 6,6′-methylene-bis-2,2,4-trimethyl-1,2-dihydroquinoline (MTDQ) and its water soluble species (MTDQ-DA) was studied in biological membrane and model systems using EPR spectroscopy for detecting molecular motion and radical formation. Both compounds influenced the rotational mobility of maleimide spin labels attached to proteins of the nerve membrane: the addition of MTDQ or MTDQ-DA induced an increase of the rigidity of the membrane in the environment of the attaching sites. The reaction of MTDQ-DA with hydroxyl and superoxide free radicals showed that this compound was also a competitive ȮH and superoxide free radical scavenger. The reaction rate constant of the formation of MTDQ-DA free radical was $k = (4.0 \pm 0.5) \cdot 10^9$ $M^{-1} s^{-1}$ in the hydroxyl free radical generating system. Simulation of EPR spectra supported that MTDQ-DA free radical was very likely a stable nitroxide free radical.

Key words: MTDQ; Free radical; Biological membrane; EPR

1. Introduction

The formation of free radicals, especially oxygen free radicals in biological systems is a consequence of aerobic life. Many studies define the molecular mechanisms of the initiation, propagation and termination of the chemical reactivity of oxy-radicals, i.e., of the lipid peroxidation.

The interest in this process rapidly increased because it has been established that the peroxidative damages of biomembranes may play an important role in number of diseases [3,18,23]. Especially, the superoxide and hydroxyl free radicals may act as etiological factors in biological phenomena under pathological conditions [8,10]. The superoxide free radical anion is highly reactive in hydrophobic environment and its attack on polyunsaturated fatty acids contributes to the initiation of lipid peroxidation [11]. The potentially toxic products of oxygen metabolism are normally removed by the ubiquitous endogenous scavengers. Nevertheless, in different diseases the overproduction of oxygen free radicals possibly combined with the reduction of scavenging mechanisms represents a dangerous state. This imbalance between oxidative reactions and protective mechanisms indicates the basis of a number of diseases and by this means the use of natural and/or artificial antioxidants could help in preventing of cell and membrane damages [30].

The peroxidation of unsaturated fatty acids of phospholipids is accompanied by alteration of the structural and functional characteristics of membranes. Lipid peroxidation and perturbations, like ionizing radiations, were found to lead to changes of the fluidity of the membranes [2,17]. Fluorescence and paramagnetic resonance studies showed that the rotational correlation times of the probe molecules increase following lipid peroxidation [1]. Considerable evidences were given that oxidizing lipids and proteins interact and free radicals are transferred to proteins [29]. In biological membranes, the conformational changes of proteins, induced by environmental factors and different drugs are presumably not attenuated immediately at the annular lipid region, but transferred via lipid-protein interactions to the bulk lipid region modifying the arrangement and mobility of the phospholipid chains [22,25,32]. In turn, the perturbation in the lipid region

*Corresponding author. Fax: +36 72 315864.

Abbrevations: MTDQ, 6,6′-methylene-bis-2,2,4-trimethyl-1,2-dihydroquinoline; DMPO, 5,5′-dimethyl-l-pyrroline-*N*-oxide; MSL, 4-maleimide-2,2,6,6-tetramethylpiperidinooxyl; EPR, electron paramagnetic resonance.

SSDI 0005-2736(93)E0317-4

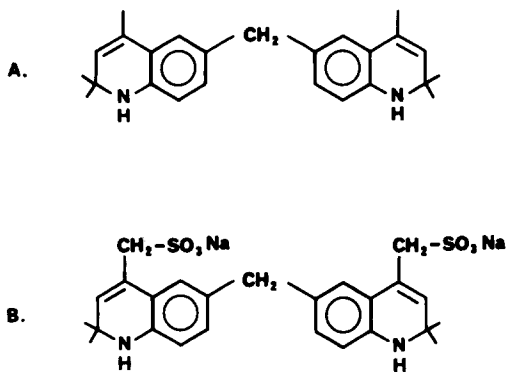

Fig. 1. Structural formulas of MTDQ compounds: (a) MTDQ; (b) MTDQ-DA.

of membranes very likely affects the packing of the annular lipids around the proteins, which leads to steric rearrangement of the protein segments and/or protein domains, thus influencing the biological functions [9,27].

The aim of the present work was to study the effect of two dihydroquinoline type radical scavengers, MTDQ and MTDQ-DA (6,6'-methylene-bis-2,2-dimethyl-4-methane sulfonic acid sodium-1,2-dihydroquinoline), in biological membrane and model systems in order to characterize their activity on molecular level (Fig. 1). We adapted the special technique of electron paramagnetic resonance (EPR) to detect changes of molecular motions in supramolecular assemblies induced by MTDQ and MTDQ-DA, and to make possible the direct identification of free radical species in different experimental situations in the presence of MTDQ.

2. Materials and methods

2.1. Sample preparation

Preparation and spin-labelling of membrane proteins. The experiments were performed on nerve membranes. The nerves (n. ischiadicus) were isolated from frog (*Rana esculenta*) and kept in Ringer's solution at 4°C. The composition of the bathing solution was 115.0 mmol NaCl, 2.5 mmol KCl, 1.8 mmol $CaCl_2$ and 2.4 mmol $NaHCO_3$ in 1000 ml of bidestilled water (pH 7.0). The nerves were spin-labelled with *N*-(1-oxyl-2,2,6,6-tetramethyl-piperidinyl)maleimide (MSL) as described earlier [9]. It is known that maleimide labels react very rapidly with thiol groups on different immobilizing sites. Therefore, the EPR spectra exhibit spectra of labels arising from both weakly and strongly immobilizing sites [13]. But pretreating the frog nerves with *N*-ethylmaleimide (NEM) at 1.0 mM concentration for 15 min before spin-labelling, it was possible to obtain EPR spectrum which reflected spin labels attached only to strongly immobilizing sites.

The first step of the labelling was the pretreatment of nerves with 1.0 mM NEM dissolved in Ringer's solution to block the thiol groups on the outer surface of the membrane. In the next step 100–200 μg of maleimide spin label (MSL) was dissolved in 10–20 μl halothane and then it was thoroughly mixed with 1.2 ml Ringer's solution at 4°C. The final concentration of label was $(3–7) \cdot 10^{-4}$ M. The nerves were immersed for 10 min in the spin-labelling solution to label the membrane proteins. During reaction the solution was carefully shaken at room temperature to achieve a high degree of labelling. After incubation the nerves were transferred to a large volume of Ringer's solution (400–500 ml) to remove the unreacted labels and the rest of halothane from the water space of the nerve by stirring gently.

Spin trapping with DMPO. The preparation and the maintenance of the reagents used for the experiments were essentially the same as described by Floyd and co-workers [6,33]. Briefly, the composition and the sequence of addition of the components were

(i) 60 μl buffer solution (100 mM NaCl, 25 mM $NaHCO_3$ (pH 7.1))
(ii) 10 μl DMPO (710 mM DMPO in water)
(iii) 10 μl ATP (10 mM ATP)
(iv) 10 μl $FeCl_2$ (1 mM $FeCl_2$ in 0.0012 M HCl)
(v) 10 μl H_2O_2 (0.3% stock solution, 8.8 mM)

The components were mixed in thermostatted water bath at 37°C for 30 s, and the last component was added and incubated for further 30 s. MTDQ-DA was dissolved in the buffer solution.

The samples were then placed into the flat cell of Zeiss (Jena, Germany) and the EPR spectrum recording was started within 3 min. In this model system hydroxyl free radicals were generated by the well-known Fenton reaction and a portion of the OH free radicals were trapped by DMPO [14,19].

Model systems. In order to investigate the properties of MTDQ, superoxide free radical generating system was used: 100 mM MTDQ-DA, 15 mM KO_2, 12 mM NaEDTA-$NaCO_3$ buffer, pH 10.2 for water soluble MTDQ [21] and KO_2-chloroform system for lipid soluble MTDQ was used, as suggested by Kanofsky [16]. The components were thoroughly mixed over ice, KO_2 was the last component added to the reaction mixture, the sample was filled in the flat cell of Zeiss and its EPR spectrum was recorded in 2 min at 22°C temperature.

2.2. EPR measurements

The EPR measurements were taken with an ERS 220 X-band spectrometer (Center of Scientific Instruments, Berlin, Germany). For conventional EPR tech-

nique 100 kHz field modulation (0.1–0.25 mT amplitude) and 10–20 mW microwave power were used. Second harmonic absorption out-of-phase spectra were recorded with 50 kHz field modulation (0.5 mT amplitude) and detection at 100 kHz out-of-phase. The saturation transfer spectra were taken with 85 mW microwave power, which corresponds to an average microwave field amplitude of 0.025 mT in the central region of the flat cell [4].

The measurements were performed at 22°C. The magnetic field calibration was made with nuclear magnetic resonance magnetometer (MJ-110 R Radiopan, Poland), the scan range was 10 or 20 mT with 13 min scan time. The free radical concentration was estimated by double integration of the EPR spectra. The sample with DMPO and a sample with known concentration MSL was measured under identical instrumental condition (microwave power 10 mW; modulation amplitude (100 kHz): 0.1 mT; scan range: 13 min with a time constant of 0.1 s).

Rotational correlation times which characterize the rotational motion of the labels were calculated using the value of the hyperfine splitting constant $2A'_{zz}$ (distance between the two outermost extrema in the EPR spectrum) and that of the sample at the rigid limit approximation according to Freed [7]. The hyperfine splitting constant at rigid limit can be obtained by an extrapolation procedure; the values of $2A'_{zz}$ were determined from the spectra at increasing viscosity and the plot was extrapolated for $\eta \rightarrow \infty$.

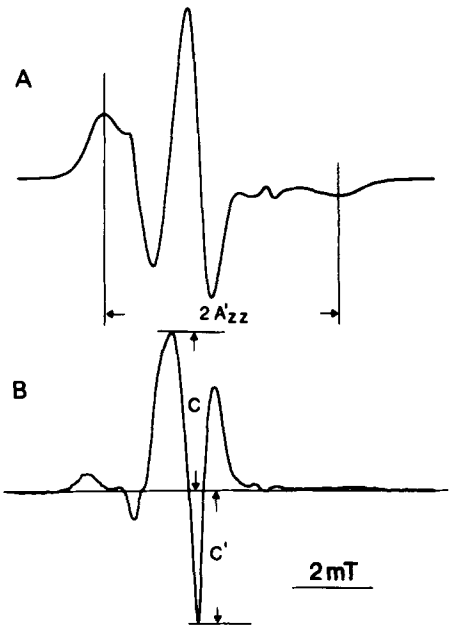

Fig. 2. Conventional (A) and saturation transfer (B) EPR spectrum of maleimide spin-labelled n. ischiadicus from frog (*Rana esculenta*). The nerve was pretreated with 1 mM *N*-ethylmaleimide for 15 min at 0°C before spin labelling with MSL. Rotational correlation time for the label estimated from the C'/C parameter of the spectrum is 0.12 ms.

3. Results and discussion

3.1. Characterization of the MSL nerves in the presence of MTDQ

The conventional and saturation transfer EPR spectra of the frog nerve are shown in Fig. 2. Spectrum A indicates that the labels were attached almost exclusively to strongly immobilizing sites. The contribution from labels located on weakly immobilizing sites could be neglected; this amount was estimated to be a few percent of the total absorption. From the EPR spectra 5.829 ± 0.025 mT was obtained for the hyperfine splitting constant. The extrapolation procedure led to the conclusion that the labels were rigidly attached to a protein domain that rotated with an apparent rotational correlation time of 62 ns. Considering the peak height ratio (C'/C) in the ST EPR spectra (spectrum B in Fig. 2.) a longer rotational correlation time $\tau_2 =$ 120 ns was obtained [31]. The difference between the two τ_2 values can be explained by a small increase of the polarity in the environment of the labelled sites during the extrapolation procedure which resulted in an under-estimate of the calculation for the rotational correlation times. Lassmann and co-workers [20] suggested an empirical scale ($h = 0$ for water and $h = 1.0$ for decaline) to characterize the microenvironment of the attaching sites by a polarity index (h) which can be derived from the hyperfine splitting constant. We obtained a value $h = 1.2$, which suggests that the labels were located in a strongly hydrophobic region of the membrane.

Both MTDQ and MTDQ-DA produced small, but significant increase of the distance between the outermost hyperfine extrema evidencing the immobilizing effect of the drugs upon the protein domain that holds the label (Fig. 3). The distance changed with increasing concentration of MTDQ, and attained to a constant level at about 20 mg/ml (about 40 mM) concentration (5.914 ± 0.025 mT). Above 20 mg/ml, only insignificant variations in the hyperfine splitting were observed. The hyperfine splitting constant for fully immobilized label ($\eta \rightarrow \infty$) was 6.013 ± 0.025 mT; the measured value suggests an increase of the rotational correlation time roughly by a factor of 2 after incubation with 20 mg/ml MTDQ.

The effect of MTDQ and MTDQ-DA cannot be compared because of the low solubility of MTDQ in water, and therefore there is a difference in the distribution of the two compounds in the frog nerve membrane.

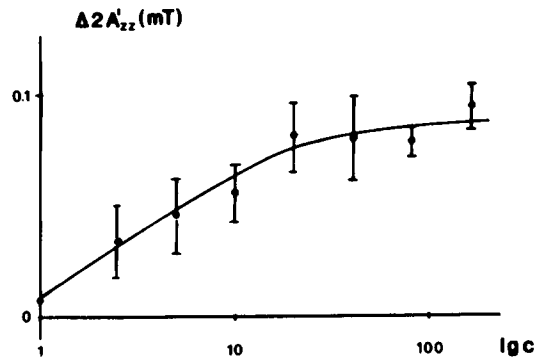

Fig. 3. Dependence of hyperfine splitting constant ($2A'_{zz}$) of EPR spectra on concentration of MTDQ-DA. The increase of $2A'_{zz}$ is plotted against MTDQ-DA concentration. The spin-labelled frog nerves were kept in Ringer's solution containing different amount of MTDQ-DA for 15 min at 4°C before spectra were taken. The number of experiments varied between 5 and 10. Standard deviations of the measurements are given as bars. The concentration of MTDQ-DA is measured in mg/ml units.

3.2. *The effect of MTDQ-DA on the hydroxyl free radical adduct of DMPO*

The EPR spectrum of DMPO samples at low concentrations of MTDQ-DA showed a 1:2:2:1 quartet with hyperfine splitting constants of $a_N = a_H = 1.48$ mT, which was identified as the DMPO-OH radical adduct (Fig. 4) [12]. At increasing concentration of MTDQ-DA in the reaction mixture the amount of DMPO-OH adduct decreased evidencing the hydroxyl free radical scavenger property of MTDQ-DA. The experimental protocol, proposed by Floyd and Lewis [6], allowed the quantitative estimation of the free radical concentration under different experimental conditions. The free radical concentration of the

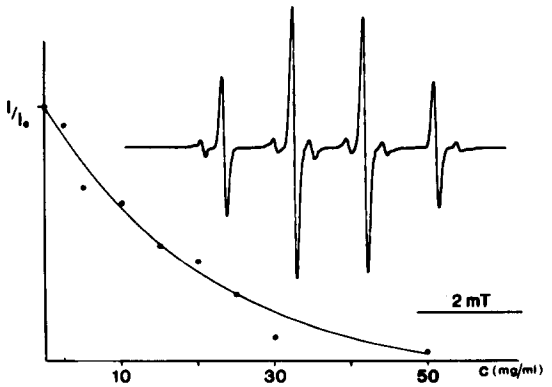

Fig. 4. Relative intensity of the EPR spectrum (second peak) of DMPO-OH adduct against the concentration of MTDQ-DA in the reaction mixture. Spectra were recorded under identical conditions, only the concentration of MTDQ-DA was varied. I_0 is the intensity of the second line in the absence of MTDQ-DA. The inset shows the EPR spectrum of the DMPO-OH adduct.

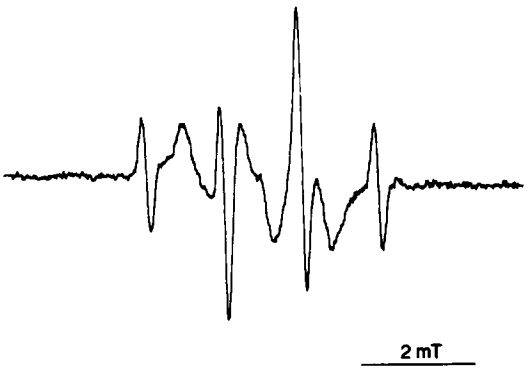

Fig. 5. EPR spectra of MTDQ-DA and DMPO-OH adduct demonstrating the hydroxyl free radical scavenger property of MTDQ-DA. In the experiment the concentration of MTDQ-DA was 70 mM, whereas the final concentration of DMPO was 71 mM in the sample.

DMPO-OH adduct at zero concentration of MTDQ-DA was about 10–20 μM. Keeping constant the amount of DMPO and H_2O_2 in the reaction mixture, the relative intensity of the EPR spectrum decreased with increasing concentration of MTDQ-DA in a non-linear manner (Fig. 4). Moreover, at higher concentrations of MTDQ-DA relative to DMPO, another free radical signal appeared in the spectrum (Fig. 5). Using the same reaction mixture and the sequence of addition of the components without DMPO, a triplet EPR signal was detected. The result of spectrum manipulation by digital subtraction strongly suggests that the chemical structure of the free radical is very likely the same which was observed in superoxide free radical generating system (Fig. 6). Varying fractions of the EPR spectrum obtained in superoxide free radical system was subtracted from the spectrum detected in hydroxide free radical generating system in absence of spin trapping substance. The residual spectrum showed only small deviations due to differences in the line-widths.

Following the procedure proposed by Schmid and Ingold [28], it was possible to estimate the rate constant for the reaction

$$\dot{O}H + \text{mtdq-da} \xrightarrow{k} \text{MTDQ-D}\dot{A} + \ldots$$

Accepting the value published by Finkelstein and co-workers [5] that $k_1 = 3.4 \cdot 10^9\ M^{-1} s^{-1}$ for the reaction

$$\text{DMPO} + \dot{O}H \xrightarrow{k_1} \text{DMPO-}\dot{O}H,$$

the best estimate for k is $(4 \pm 0.5) \cdot 10^9\ M^{-1} s^{-1}$. The reaction scheme shows that MTDQ-DA reacts with the hydroxyl free radical at a rate comparable to the rate of the DMPO-OH radical adduct formation. Therefore, it can be suggested that MTDQ-DA represents a competitive radical scavenger compound in the reaction.

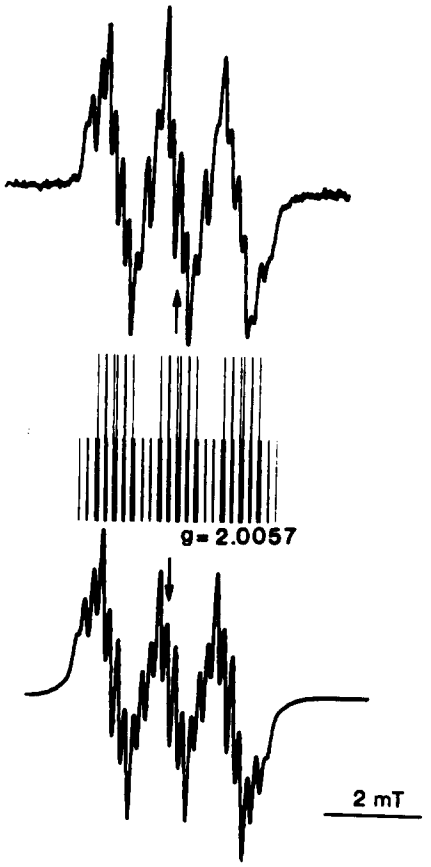

Fig. 6. EPR spectrum of MTDQ-DA in superoxide free radical generating system (100 mM MTDQ-DA, 15 mM KO_2, in 25 mM NaEDTA-Na_2CO_3 buffer, pH 10.2). The EPR spectrum was taken starting within 2 min after the addition of the last component to the reaction mixture. Simulated EPR spectrum of the MTDQ-DA free radical (bottom). The hyperfine splitting constants were $a_N = 1.142$ mT, $a_H = 0.348$ mT and $a_H = 0.318$ mT, $a_H = 0.153$ and $a_H = 0.143$ mT. The individual linewidth was 0.12 mT and supposed to be Lorentzian. In the middle the stick spectrum is shown.

3.3. MTDQ-DA in superoxide free radical generating systems

It was shown that KO_2 in alkaline buffer yields superoxide free radical anion, $\dot{O}_2^-$ [15]. The result obtained when MTDQ-DA was added to the system is shown in Fig. 6. The appearance of strong EPR signal immediately after addition of MTDQ-DA to the reaction mixture demonstrates clearly the interaction of MTDQ-DA with the superoxide free radicals which leads to formation of stabil radical product(s). The well-resolved superhyperfine structure on the triplet hyperfine structure might arise from an odd electron located at a nitroxide group and interacts with ring protons. The finding shows that MTDQ-DA, as an active species, participates in the free radical reaction, and might act as a synthetic radical scavenger in the superoxide free radical anion generating systems.

Using MTDQ in apolar solvent (chloroform or dimethyl sulfoxide) the same EPR spectrum was detected, only the resolution of the superhyperfine structure was significantly diminished.

3.4. The MTDQ-free radical

The existence of basic triplet in the EPR spectrum with hyperfine splitting constant of 1.142 ± 0.025 mT in water solution indicated that the free radicals were rather nitroxide free radicals than those of nitroso-aromatic compounds, which have significantly smaller hyperfine splitting constants [24]. Very likely, the interaction of $\dot{O}_2^-$ with MTDQ-DA in alkaline solution resulted in a reaction involving the addition of the protonated form of the superoxide free radical anion to the amine nitrogen followed by the loss of water which led to the formation of nitroxide. This mechanism is similar to that suggested earlier in hydroxyl free radical generating system [33].

In order to assign a more precise structure to the free radical giving rise to the EPR spectrum, simulated spectra were compared with the experimental spectra. The best fit with the observed EPR spectra was achieved using the following hyperfine coupling constants (Fig. 6):

$a_N = 1.142$ mT N-1

$a_H = 0.348$ mT C-3

$a_H = 0.318$ mT C-7, C-8

$a_H = 0.153$ mT C-5

$a_H = 0.143$ mT

The unpaired electron interacts with the nitrogen nucleus and the ring proton at the C-3 atom; even more the unpaired electron has also significant spin density at the protons of the C-5, C-7 and C-8 atoms. The spin densities on the latter two carbon atoms were supposed to be equal. However, proton magnetic resonance spectra exhibited that the ring protons at carbon atoms in positions 7 and 8 were only nearly equivalent ($\delta = 6.57$ and 6.85 ppm with respect to DDS standard), for the ring proton at the fifth carbon atom the chemical shift was 7.18 ppm and at C-3 5.74 ppm. The smallest contribution to the superhyperfine structure is expected from the methylene protons.

It should be noted that the dependence of linewidth on nuclear spin orientation was not included in the simulation procedure.

3.5. Concluding comments

Under pathological conditions the increase in free radical activity represents an important factor in the injury of cells and membranes. Therefore, the idea of therapy with artificial antioxidant agents seems to be

logic step to substitute for the imbalanced scavenger states.

A major, not completely answered question about free radicals is their reactivity with cell components in vivo. It is exactly not known, to what extent the free radicals react with biomolecules, and how important the free radical pathways are and how they are influenced by administration of antioxidants. New technology is needed for measuring of free radical species within membrane enclosed domains. EPR method applied for this study may be a suitable experimental strategy. According to our model experiments the quantification of free radical generation could be followed in steps and could effectively be influenced by manipulation of MTDQ derivates.

According to our basic studies with EPR, we can conclude that

(i) both MTDQ and MTDQ-DA decrease the mobility of the maleimide spin label attached to the thiol sites of the membrane proteins in the sciatic nerve prepared from frog. It might be supposed that the changes in the mobility of the protein domains could influence the chemical reactions and transport processes which are responsible for the physiological activity;

(ii) the experimental results in hydroxyl free radical generating system proved that MTDQ-DA was a strong OH free radical scavenger, and the reaction rate constant of the formation of MTDQ-DA was comparable with that of the DMPO-OH adduct;

(iii) evidence was obtained in superoxide free radical generating system that MTDQ in hydrophobic environment, and MTDQ-DA in aqueous solution participated in the free radical reactions. It can be assumed that the presence of MTDQ in the cell membranes may improve the protection of membrane components against free radical attacks.

4. Acknowledgements

The work is part of the research project supported by grants of the Hungarian Scientific Research Foundation (OTKA No. 2044 and No. 1577). The authors thank Dr. Hideg for supplying the maleimide spin label.

5. References

[1] Barrow, D.A. and Lents, B.R. (1981) Biochim. Biophys. Acta 645, 17–23.
[2] Bruch, R.C. and Thayer, W.W. (1983) Biochim. Biophys. Acta 733, 216–222.
[3] Fantone, J.C. and Ward, P.A. (1982) Am. J. Pathol. 107, 395–418.
[4] Fajer, P. and Marsh, D. (1982) J. Magn. Reson. 49, 212–224.
[5] Finkelstein, E., Rosen, G.M. and Rauckman, E.J. (1980) J. Am. Chem. Soc. 102, 4994–4999.
[6] Floyd, R.A. and Lewis, C.A. (1983) Biochemistry 22, 2645–2649.
[7] Freed, J.H.(1976) in Spin Labeling, Theory and Application (Berliner, L.J., ed.), Vol. 1, pp. 83–91, Academic Press, New York.
[8] Friedovich, I. (1983) Annu. Rev. Pharmacol. Toxicol. 23, 239–257.
[9] Gróf, P. and Belágyi, J. (1983) Biochim. Biophys. Acta 734, 319–328.
[10] Halliwell, B. and Gutteridge, J.M.C. (1984) Biochem. J. 215, 1–14.
[11] Halliwell, B. and Gutteridge, J.M.C. (1986) Arch. Biochim. Biophys. 246, 501–514.
[12] Harbour, J.R., Choow, V. and Bolton, J.R. (1974) Can. J. Chem. 52, 3549–3554.
[13] Jost, P., Brooks, U.J. and Griffith, O.H. (1973) J. Mol. Biol. 76, 313–318.
[14] Jansen, E.G. (1980) in Free Radicals in Biology (Pryor, W.A., ed.), Vol. 4, pp. 115–154, Academic Press, New York.
[15] Kanofsky, J.R. (1983) J. Biol. Chem. 258, 5991–5993.
[16] Kanofsky, J.R. (1986) J. Am. Chem. Soc. 108, 2977–2979.
[17] Koter, M. and Surewitz, W.K. (1985) Stud. Biophys. 106, 79–86.
[18] Kramer, J.H., Arroyo, O.M., Dickens, B.F. and Weglicki, W.B. (1987) Free Radic. Biol. Med. 1, 153–159.
[19] Lai, C. and Piette, L.H. (1977) Biochem. Biophys. Res. Commun. 78, 51–59.
[20] Lassmann, G., Ebert,B., Kuznetsov, A.N. and Damerau, W. (1973) Biochim. Biophys. Acta 310, 298–304.
[21] Marklund, S. (1976) J. Biol. Chem. 251, 7504–7507.
[22] Migazawa, T. (1985) J. Mol. Struct. 126, 493–502.
[23] Morrison, H., Jernstrom, B., Nordenskjold, M., Thor, H. and Orrenius, S. (1984) Biochem. Pharmacol. 33, 1763–1769.
[24] Ozawa, T. and Manaki, A. (1986) Biochem. Biophys. Res. Comm. 136, 657–664.
[25] Quinn, D.J. and Chapman, D. (1980) CRC Crit. Rev. Biochem. 8, 1–117.
[26] Rodgers, M.A.J. and Powers, E.L. (1981) Oxygen and Oxy-Radicals in Chemistry and Biology, pp. 197–204, Academic Press, New York.
[27] Seki, H., Ilan, Y.A., Ilan, Y. and Stein, G. (1976) Biochim. Biophys. Acta 440, 573–586.
[28] Schmid, P. and Ingold, K.U. (1978) J. Am. Chem. Soc. 100, 2493–2500.
[29] Schaich, K.M. and Karel, M. (1976) Lipids 11, 392–400.
[30] Slater, T.F. (1984) Biochem. J. 222, 1–15.
[31] Thomas, D.D., Dalton, L.R. and Hyde, J.J. (1976) J. Chem. Phys. 65, 3006–3024.
[32] Vanderkooi, G., Shaw, J., Storms, E., Vennerstrom, R. and Chignell, D. (1981) Biochim. Biophys. Acta 635, 200–203.
[33] Zs.-Nagy, I. and Floyd, R.A. (1984) Biochim. Biophys. Acta 790, 238–250.