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Time course of the age-related alterations in stored blood

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Abstract

The extent and time course of the impairments occurring in whole blood and erythrocyte cells stored under blood bank conditions were studied by monitoring the reduction of MAL-6 spin label added to the media containing whole blood or erythrocyte cells using electron spin resonance (ESR) technique. Impairments forming in the erythrocyte cells incubated for various times at 37 °C were also studied. Erythrocyte cells were found to undergo changes during the storage or incubation, leading to fast decay of MAL-6 spin labels signal height. The extent of the changes depends on storage or incubation time. However, the reduction in incubated or artificially aged erythrocyte (AAE) cells was faster than the reduction in whole blood (WB) and aged erythrocyte (AE) cells stored under blood bank conditions. Two exponential curves attributed to the liquid and cellular parts of a given samples were found to be described best in the reduction of MAL-6 spin label in WB, AE and AAE.

Keywords: Erythrocyte; Aging; Nitroxide; ESR

1. Introduction

When blood is collected in a standard anticoagulant preservative solution and stored under blood bank conditions the red cells slowly undergo changes, which lead to a loss of red cell viability and the capacity to off-load oxygen normally. The red cell viability and hence its post-transfusion survival is related in large extent to the structural and metabolic status of the red blood cell membrane. Spin-label methodologies have been extensively applied to monitor alterations in the behavior of membrane protein and phospholipids [1–10]. Stearic acid and MAL-6 (4-maleimide-2,2,6,6-tetramethyl-piperidinyl-1-oxyl) spin labels have been exclusively used to study the properties of the erythrocyte membrane.

Besides its quantitative reactions with cell proteins such as spectrin, band 3, band 2-1 and other high molecular weight proteins [7,11–13] MAL-6, like other nitroxide may undergo significant redox reactions in biological systems [14–21] which is pronounced as a decrease of the ESR signal intensity due to the loss of its paramagnetism. Erythrocytes stored under blood bank condi-

Abbreviations: WB, Whole blood; AE, Aged erythrocyte; AAE, Artificially aged erythrocyte; ESR, Electron spin resonance.

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tions undergo progressive metabolic impairment originating from profound rearrangement of the membrane components, which gives rise to the production of vesicles [22–24]. Moreover, the degradation of erythrocyte membrane proteins by exogenous or endogenous proteinases has been shown to affect substantially the order of the membrane and hence, the ESR signals of the spin labels attached to the protein components of this membrane [7,11,14,19,21,25,26].

Characterization and determination of the rate of the alterations occurring in erythrocyte cells during the storage are two important aspects of the blood storage. Although, the alterations in shape and membrane structure of erythrocyte cell have been extensively studied [1–10,14,17–21], the rate of these alterations are studied only in few works [1,4]. Therefore, the aim of the present work is to study in detail the rate of the alterations occurring in whole blood in erythrocyte suspensions stored in blood bank conditions and in erythrocyte suspensions artificially aged at 37 °C basing on the redox reactions of MAL-6 spin label in these media by ESR spectroscopy.

2. Materials and methods

2.1. Sampling of blood, storage and incubation

Human blood was collected by venipuncture into heparinized tubes from type A Rhesus positive volunteers. Immediately after collection, blood was subdivided into two parts. The first was stored at +4 °C under blood bank conditions and it is called as stored whole blood (WB). The second was used to get intact erythrocyte cells, which were sedimented by centrifugation of whole blood at $1100\times g$ for 20 min. After collecting the buffy coat by aspiration, the packed erythrocytes were suspended in the phosphate-buffered ringer solution (PBS) composed of 140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, 0.9 mM $CaCl_2$ and 0.5 mM MgCl. Complete removal of platelets and leukocytes from the erythrocyte suspension was obtained by repeating this procedure three times. Washed intact erythrocytes were also divided into two parts. The first part was stored at +4 °C under blood bank conditions and the second part was incubated at 37 °C with gentle shaking for predetermined times. They were called aged erythrocyte (AE) and artificially aged erythrocyte (AAE), respectively. All preparations were performed under steril conditions; nevertheless 200 μg ml^{-1} Avocin was added to the incubation mixture. Sterility tests were performed throughout the experiments and proved to be negative. Measurements were carried out on 1 ml aliquots of WB, AE and AAE at the end of predetermined storage (up to 60 days) and incubation (up to 73 h) times after adding appropriate amount of MAL-6 (4-maleimide-2,2,6,6-tetramethyl-piperidinyl-1-oxyl) spin label.

2.2. Method and measurements

Signal intensity decays of MAL-6 spin label in WB, AE and AAE samples were monitored using ESR spectroscopy. MAL-6 spin label was purchased from Sigma and used without further purification. 0.1 ml of 1 mM MAL-6 chloroform solution was evaporated by nitrogen gas flow and solvent was completely removed by continous pumping for 12 h. Then, 2 ml blood sample was added on and the mixture was shaked gently for 2 min. Measurements were performed on the samples transfered into calibrated capillary tubes placed in the normal ESR tubes of 4 mm inner diameter permanently positioned in the resonance cavity. Each experiment was repeated three times and the results were given as the mean of these measurements. The intensity of ESR signals was calculated using the formula $I=\Delta H_{PP}^2\times I_{PP}$ where ΔH_{PP} and I_{PP} are the linewidth and height of the mid-field hyperfine line of MAL-6 spectrum, respectively. The decrease in the signal intensity of MAL-6 spin label as a function of time was monitored to carry out kinetic information concerning the interactions between MAL-6–whole blood and MAL-6–erythrocyte cells stored under blood bank conditions or artificially aged for various time lengths. Five minutes after the addition of whole blood or erythrocyte PBS suspensions to the beackers containing spin label was adopted as measurement starting point for each sample and measurements were performed every 5 min over a period of 120 min. All signal

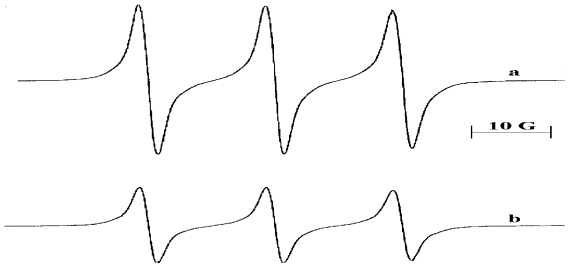

Fig. 1. ESR lineshape of MAL-6 spin label in fresh WB. (a) 10 min; (b) 120 min after the addition of spin label.

intensities were normalized with respect to the initial signal intensity.

A Varian 9' E-L X band ESR spectrometer operating at 9.5 GHz and equiped with a TE_{104} rectangular double cavity was used to carry out the measurements. All spectra were recorded at room temperature (approx. 20 °C). A DPPH reference sample placed in the front cavity was used to control spectrometer setting conditions from one sample to another.

3. Results

3.1. Stored whole blood (WB)

ESR line shape of MAL-6 in fresh WB 10 and 120 min after the addition of the spin label are shown in Fig. 1. As seen, both of the spectra are typical of dilute MAL-6 nitroxide aqueous solutions. That is, the line shape excludes any evidence of strongly immobilized spin label. Therefore, it can be concluded that even 120 min after the addition, spin labels stay always in the liquid environment of the blood and that no attachment of the spin labels to the blood cells takes place during the whole measurement time (0–120 min).

ESR line shape of MAL-6 in WB stored for various times was found to stay unchanged also during the measurement time. As for the signal intensity, it is decayed with a rate depending on the storage period over the measurement time. The decay in signal intensity in WB is expected to result from rapid redox processes involving the MAL-6 spin label in the presence of different blood components. Experiments were performed to determine the contribution of the plasma part to the redox processes of MAL-6. Plasma isolated from fresh and stored WB containing appropriate amount of MAL-6 spin label were used in these experiments. The results obtained for a plasma sample isolated from WB stored for 60 days is given in Fig. 2. As seen, signal intensity varies very slowly over the measurement time. This was also the case for plasma isolated from fresh blood. Therefore, it was concluded that MAL-6 involving redox processes in fresh and stored WB arise rather from cellular part, that is from the part including erythrocytes, leucocytes and thrombocytes of the blood. This fact avoid us to perform a reliable comparison of the signal intensity decays of two different samples due to the difficulty in controlling the amount of plasma and cellular parts in a given sample and the volumes that they occupy in the effective length of the microwave cavity. To overcome this difficulty a fitting procedure was adopted assuming that MAL-6 spin labels staying in the plasma part and not interacting with the cell components of the blood contribute very weakly to the signal intensity decay while those spin labels staying in the vicinity of erythrocyte, leucocyte and thrombocytes dominates the signal intensity decay. Although the spin label concentrations were same for studied WB samples, their

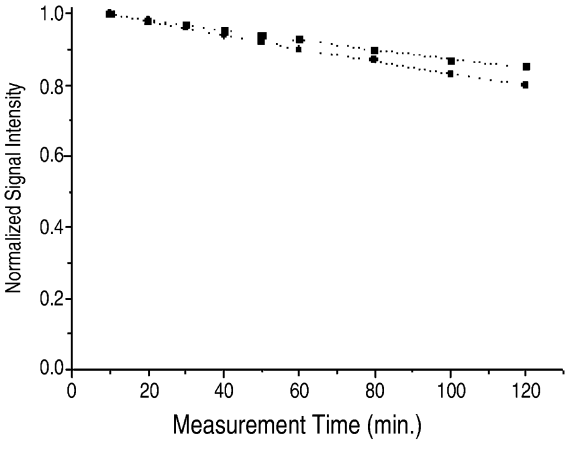

Fig. 2. Variation of the mid-line intensity of MAL-6 spin label during the measurement time in the fresh plasma and in plasma isolated from WB stored for 60 days. (■) fresh, (●) stored. Symbols: experimental; doted lines: theoretical.

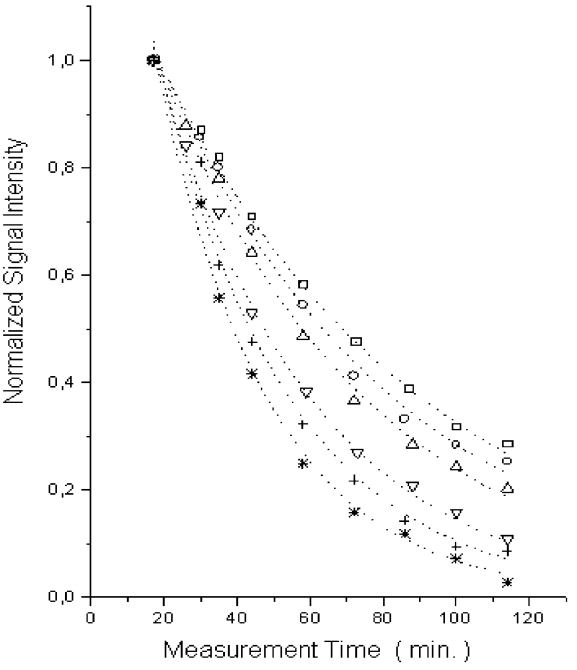

Fig. 3. Variation of the mid-line intensity of MAL-6 spin label in fresh and stored WB over the measurement time. Storage days at blood bank conditions are: (□) fresh, (○) 5, (△) 15, (▽) 20, (+) 30 and (*) 60. Symbols: experimental; doted lines: theoretical.

plasma and cellular parts differed from one sample to another. Therefore, the experimental signal intensity data of each sample were fitted to two exponential decay functions of different decay constants and of different weights. Exponential decay functions obtained from fitting procedure and corresponding experimental signal intensity decay data both normalized to their values at measurement starting times, that is, 10 min after the addition of MAL-6 spin label, are given in Fig. 3 for WB samples stored for 0; 5; 15; 20; 30; 30 and 60 days. As seen, the decay rate of mid-field line intensity of MAL-6 spin label increases with increase in storage time. The decay constants calculated for studied samples are summarized in Table 1.

3.2. Aged intact erythrocytes (*AE*)

The decays of MAL-6 spin label signal intensity in the PBS suspension of erythrocyte were also studied using ESR spectroscopy. The possible decay of MAL-6 spin label in pure PBS solution was investigated first and, as in the case of plasma, a very slow exponential decay was observed (Table 1). However, the presence of intact erythrocyte in the suspension was found to produce a rapid decay in the signal intensity of MAL-6 spin label. Therefore, it is concluded that spin labels staying in very close vicinity of erythrocyte cells undergo rapid redox processes which reduce the spin labels to ESR silent units as in the case of stored WB. A similar fitting procedure that is used in Section 3.1 was adopted to analyse the experimental signal decay data obtained for AE samples. The experimental and theoretical data derived are given in Fig. 4 and Table 1. As in the case of stored WB, the longer the storage time the faster is the decay of MAL-6 spin label signal intensity.

3.3. Artificially aged intact erythrocytes (*AAE*)

The kinetics of the redox reactions that MAL-6 spin label is involved in the PBS suspension containing intact erythrocyte cells incubated at 37 °C in protein free medium for various times were investigated in this section. The results obtained using similar data analysis procedure discussed in the previous sections are given in Fig. 5 and Table 1. The main conclusion that one can draw from these data is, longer the incubation time, faster is the decay rate of MAL-6 spin label, in AAE suspensions.

4. Discussion

The investigation carried out in the present work seeking the determination of the evolution of the impairments occuring in the bloods stored under blood bank conditions or artificially aged is based on the direct measurements of loss of paramagnetism of MAL-6 spin label added to the samples prepared from those blood by monitoring ESR signal intensity. However, apparent decreases in spin label concentration may also occur from the possible changes of spectroscopic factors rather than true redox processes of nitroxide radical. This is especially likely, if the concentration of spin lable is determined by measurements of line

Table 1
Decay constants calculated from fitting procedure for studied samples

Samples	Storage or incubation time	Decay constant[a] $k\times10^3$ (min^{-1})	Correlation coefficient r
Plasma	Fresh	1.52±0.43	0.601
	60[b]	2.06±0.76	1.003
PBS solution	Fresh	1.32±0.27	0.987
WB	Fresh	14.54±1.76	0.743
	5[b]	15.84±1.17	0.861
	15[b]	17.97±1.53	0.729
	20[b]	24.37±2.05	0.854
	30[b]	27.65±2.47	0.916
	60[b]	32.85±3.15	0.943
AE	Fresh	20.40±1.67	0.922
	15[b]	22.35±1.73	0.891
	30[b]	29.29±2.11	0.998
	60[b]	32.26±3.22	0.910
AAE	Fresh	20.40±1.67	0.691
	19[c]	35.30±3.50	0.799
	43[c]	40.48±4.10	0.916
	73[c]	67.29±4.89	0.677

WB: Whole blood.
AE: aged erythrocyte stored under blood bank conditions.
AAE: erythrocyte artificially aged at 37 °C in protein free medium.
[a] Decay constants are expressed as means of three independent measurements ± S.D.
[b] Storage time in days.
[c] Incubation time in hour.

heights, which is the case in the present work, because several factors can simultaneously affect line width. Relatively small changes in line width can lead to large changes in the line height. The decrease in motional amplitude and increase in oxygen concentration can result in line broadening. The lineshapes and line widths of the ESR spectra recorded for samples studied in the present work were found to stay unchanged under the same experimental conditions during ESR measurement time (120 min), so that the kinetic constants can be directly obtained by plotting ln(line height) vs. measurement time. The independence of line width from time allowed us to exclude the possible line broadening effect originating from the changes in the O_2 concentration in the studied samples during the course of ESR measurements. The shapes of the spectra obtained for the studied samples indicate that MAL-6 spin label is located in the aqueous parts of the samples without penetrating into the membranes of constituent cellular elements.

MAL-6 has the nitrogen atom of the nitroxide group in an unsaturated heterocyclic ring. The kinetic stability of the nitroxide group is primarily based on steric blocking via bulky methyl groups on the adjacent ring carbons, although electron delocalization also plays a significant role. MAL-6 losts its paramagnetism through many different reactions [27,28]. However, recombination with free radicals and reduction through the capture of an electron and a proton are two main reactions encountered in biological systems.

Nitroxides are quite stable in aqueous solutions, especially at alkaline pH [28–30]. However, detectable losses of very stable nitroxides even in distilled water have been reported [31]. The weak losses in the MAL-6 spin label paramagnetism

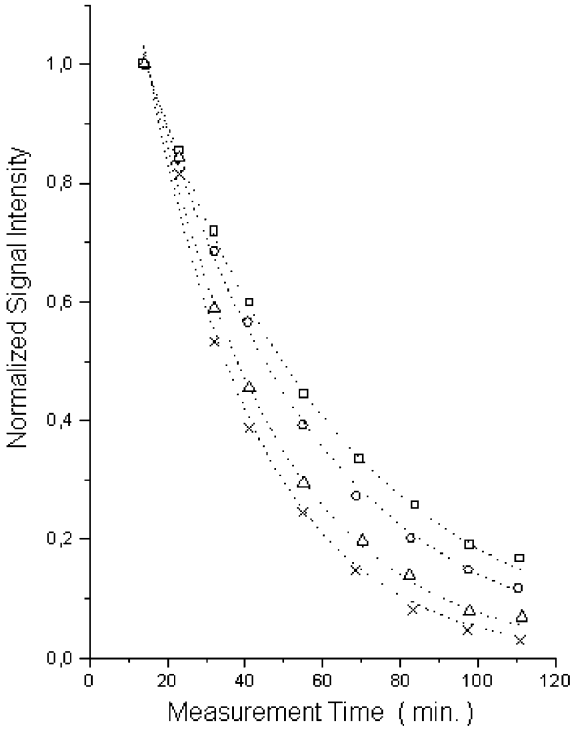

Fig. 4. Variation of the mid-line intensity of MAL-6 spin label in the PBS suspension of fresh and stored intact erythrocyte (AE) over the measurement time. Storage days at blood bank conditions are: (□) fresh, (○) 15, (△) 30 and (x) 60. Symbols: experimental; doted lines: theoretical.

observed in the present work in PBS and blood plasma reflects the presence of electron sources in these media (k for PBS $= 0.00132 \pm 0.00027$, k for plasma $= 0.00152 \pm 0.00043$). The presence of one or two electrons donating centers in the medium gives rise to the formation of sterically hindered hydroxylamines [32]. However, a feature of nitroxide reduction is that the process takes place only if both an electron donor and a proton donor are present in the medium. Therefore, the reduction of nitroxides undergoes only in an acid organic medium or water. However, the samples studied in the present work contain microheterogeneities and as a result of this all samples have aqueous and cellular parts. Transport and reduction reaction characteristics in these parts are very different. Therefore, the localization of nitroxide in the heterogeneous medium can change drastically the kinetics of reduction of nitroxides [33].

Biexponential signal decay kinetics is observed in the present work for the studied blood samples, because it is normally indicative of different regions of localization of nitroxides (aqueous solution and vicinity of cellular elements). Nitroxides with maleimide can react with SH group of proteins [17,34,35]. It was shown that the incubation of nitroxides in native erythrocytes and platelets resulted in the reduction of nitroxide moiety, and it was assumed that the reaction was connected with SH group both inside the cells and in the blood plasma [18–20]. Reduction also may be determined by the simultaneous presence of two or more different reducers with different localization, e.g. by direct interaction of nitroxides with sulfhydryl groups of peptides and proteins and by

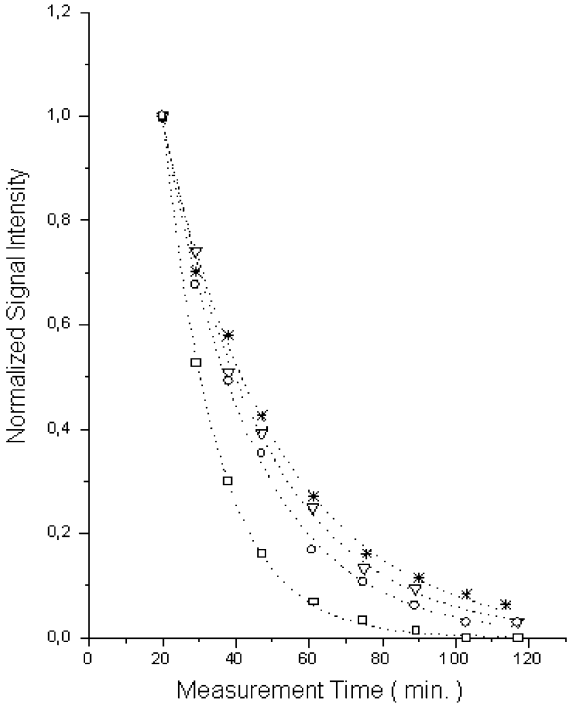

Fig. 5. Variation of the mid-line intensity of MAL-6 spin label in the PBS suspension of fresh and incubated intact erythrocyte (AAE) over the measurement time. Incubation temperature and times expressed in hours were, respectively, 37 °C and (*) fresh, (▽) 19, (○) 43 and (□) 73. Symbols: experimental; doted lines:theoretical.

reduction and functioning of the electron transport chain in biological membranes. Ascorbic acid is another component in erythrocytes, which can reduce directly or mediators nitroxides [36], although its activity in a membrane suspension is reduced in comparison with that of cells.

Red cells undergo a large number of changes in shape and membrane structure during storage under blood bank conditions and/or incubation at 37 °C. Discocyte–echinocyte shape changes and cellular swelling takes place just at the begining, then spectrin rearrangement and dephosphorylation of proteins occur. After many intermediary events it changes and reaches to the step where irreversible membrane crosslinking and loss of lipid vesicles take place [22]. The differential consumption of MAL-6 spin label calculated in the present work by ESR technique is indicative of an increasing susceptibility to oxidative impairment of blood systems. Experimental results show that longer the storage time and/or incubation time the bigger is the impairment in WB, AE and AAE and, therefore, is faster in the decay of signal intensity (Table 1). The decay rates of nitroxide increase with increase in storage time for both AE and WB and they reach to 0.03226 ± 0.00322 and 0.03285 ± 0.00315 at the end of 60 days storage, respectively. As for impairment in the erythrocyte incubated at 37 °C, it is much faster. Rate constant for AAE samples reaches to a value of 0.06729 ± 0.00489 after an incubation period of 60 h. This is nearly two times bigger than the rate constant obtained for AE and WB samples at the end of a storage time of 60 days. This finding is in accordance with the results given in literature [4,37]. Increase in vesicles release of erythrocyte with the increase in storage and/or incubation time may represent one of the major increase in MAL-6 spin label consumption due to an increase in the number of accessible membrane protein sulfhydryl groups (SH). However, the crenation depends on the lipid bilayer and on the membrane skeleton, which plays a critical role in the maintenance of red cell integrity and shape.

In accordance with our experimental results, erythrocytes incubated in protein-free media have been found to undergo a rapid process of aging with loss of approximately 50% of sialoglycopeptides from the membrane [25]. In vivo aged erythrocytes also have been observed to loose their membrane lipids and carbohydrates imposing the rearrangement of the membrane components. This produces alterations in the states of membrane proteins, so that the access of MAL-6 spin label to their sulfhydryl groups becomes easier.

5. Conclusions

We conclude from the results of the present work and relevant data given in the literature that:

1. Biexponential decay approach better describes the experimental nitroxide signal intensity decay data obtained for WB, AE and AAE samples;
2. impairment in erythrocytes incubated at 37 °C is much faster than the erythrocytes stored at blood bank conditions;
3. variation of impairment with storage time in AE and WB are not very different, and, therefore, the majority of impairment originates from erythrocyte cells;
4. sulfhydryl groups in blood samples are primarily responsible for the reduction of nitroxide free radicals;
5. oxidative damage of membrane may leads to drastic changes in the structure and function of stored and artificially aged erythrocytes; and
6. the efficiency of various blood preservative can be studied by this technique.

References

[1] G. Bartosz, BBA 664 (1981) 69.
[2] M.A. Foster, Brit. J. Haematol. 47 (1981) 1.
[3] L.W.M. Fung, Ann. N.Y. Acad. Sci. 414 (1983) 162.
[4] G.P. Pessina, L. Ciccoli, M.P. Picchi, F. Laschi, G. Fanetti, Z. Naturforsch 44C (1989) 824.
[5] T. Shiga, N. Maeda, T. Suda, K. Kon, M. Sekiya, BBA 553 (1979) 84.
[6] M.J. Barber, G.M. Rosen, E.J. Rauckman, BBA 732 (1983) 126.
[7] B.T. Farmer, T.M. Harmon, D.A. Butterfield, BBA 821 (1985) 420.
[8] Ö. Korkmaz, M. Korkmaz, Biophys. Chem. 69 (1997) 167.
[9] Ö. Korkmaz, Biophys. Chem. 83 (2000) 111.
[10] P. Copper, J. Kudynska, H.A. Buckmaster, R. Kudynski, BBA 1139 (1992) 70.

[11] G. Bartosz, M. Gaczyńska, BBA 821 (1985) 175.
[12] L.W.M. Fung, M.J. Simpson, FEBS Lett. 108 (1979) 269.
[13] D.A. Butterfield, in: L.J. Berliner, J. Reuben (Eds.), Biological Magnetic Resonance, 4, Plenum Press, London, New York, 1982, p. 1.
[14] G.J. Giotta, H.H. Wang, B.B. Res. Comm. 46 (1972) 1576.
[15] N. Kocherginsky, H.M. Swartz, Nitroxide Spin Labels Reactions in Biology and Chemistry, CRC, London, 1995, p. 96.
[16] M. Esmann, P.C. Sar, K. Hideg, D. Marsh, Anal. Biochem. 213 (1993) 336.
[17] P.R. Van Iwaarden, A.J.M. Driessen, W.N. Konings, BBA 1113 (1992) 161.
[18] C. Chagalj, T.C.P. DePaoli, A.A. Hager, L.A Palaoro, E.R. De Cellis, H.A. Farach, et al., Nuovo Cimento 4d (1984) 245.
[19] A.C. Ruggiero, N.C. Meirelles, M. Tabak, Stud. Biophy. 109 (1985) 101.
[20] P. Bette-Bobillo, A. Luxembourg, A. Bienvenue, BBA 860 (1986) 194.
[21] K. Chen, M.G. McLaughlin, BBA 845 (1985) 189.
[22] L.C. Wolff, Transfusion 25 (1985) 185.
[23] S.L. Schrier, P.R. Sohmer, G.L. Moore, L. Ma, I. Junga, Transfusion 22 (1982) 261.
[24] G.P. Pessina, V. Bocci, L. Paulesu, C. Alessandrini, R. Gerli, J. Submicr. Cytol. 12 (1980) 311.
[25] V. Bocci, G.P. Pessina, L. Paulesu, Life Sci. 24 (1979) 1351.
[26] E. Beutler, C. West, K.G. Blume, J. Lab. Clin. Med. 88 (1976) 328.
[27] B.J. Gaffney, The Chemistry of Spin Labels, in: L.J. Berliner (Ed.), Spin Labeling: Theory and Application, 1, Academic Press, New York, 1976, p. 184.
[28] E.G. Rozantsev, Free Nitroxyl Radicals, Plenium Press, New York, 1970, p. 970.
[29] G.J. Giotta, H.H. Wang, B.B. Res. Comm. 46 (1972) 1576.
[30] T. Kagiya, R. Kimura, C. Komura, K. Sakano, S. Nishimoto, Chem. Lett. 1471 (1983) 3.
[31] L.R. Brown, K. Wuthrich, BBA 464 (1977) 356.
[32] M.E. Brik, Synth. Commun. 20 (1990) 3283.
[33] D.A. Chapman, G.J. Killian, E. Gelerinter, M.T. Jarrett, Biol. Reprod. 32 (1985) 884.
[34] M. Esmann, P.C. Sar, K. Hideg, D. Marsh, Anal. Biochem. 213 (1993) 336.
[35] V.V. Khramtsov, V.I. Elinova, T.E. Goryunova, L.M. Weiner, Biokhimiya 56 (1991) 1567.
[36] Y. Zhang, L.W.M. Fung, Free Rad. Biol. Med. 16 (1994) 215.
[37] M. Sentjure, D.V. Apte, L. Mac Allister, H.M. Swartz, Curr. Top. Biophys. 18 (1994) 81.