Spin Label Studies of Erythrocytes during Storage of Blood

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The kinetic behavior of the spin label MAL-6 in the interaction with differently aged human erythrocyte membranes was evaluated by monitoring the rate of disappearance of the room temperature ESR signal due to the MAL-6 spin label added to blood after storage at 4 °C or after incubation of red cells at 37 °C in a protein-free medium. After 35 days of blood storage or 60 h of erythrocytes incubation at 37 °C the decrease of the intensity of the MAL-6 ESR spectra in respect to control samples is markedly enhanced and the correspondent kinetic constants significantly increase. Signal decay of MAL-6 is a further proof that during storage of blood under blood bank conditions or during an artificial ageing of erythrocytes at 37 °C, profound modifications occur in the human erythrocyte membrane.

Introduction

Spin-label methodologies have been extensively applied to monitor changes in the behavior of membrane [1] proteins and phospholipids [2]. In particular the spin label MAL-6 (4 maleimide-2,2,6,6-tetramethyl-piperidinyl-1-oxyl) has been widely used in the study of the properties of the erythrocyte membrane because it is known that MAL-6 quantitatively reacts with cell proteins such as spectrin, band 3, band 2–1 and other high molecular weight proteins [3]. Moreover the MAL-6, like other nitroxides [4], may undergo significant redox reactions in living systems; as a consequence of this fact loss of paramagnetism can be detected as a decrease of the ESR signal intensity.

Recently it has been reported [5] that degradation of the erythrocyte membrane proteins, induced by exogenous or endogenous proteinases, can substantially affect the ESR spectra. Moreover it is well known that erythrocytes stored under blood bank conditions undergo progressive metabolic impairment [6–8] and a profound rearrangement of the membrane components with production of vesicles [9].

In this paper we report on the interaction of MAL-6 with human erythrocytes under different experimental conditions in terms of X-band ESR lineshape and of the rate of disappearance of the ESR signal intensity from blood and washed erythrocyte suspensions during storage under blood bank conditions, or during incubation of red cells at 37 °C in a protein free medium.

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Materials and Methods

Sampling of blood, storage and incubation

Human blood, drawn from type A Rhesus positive volunteers, was collected in citrate-phosphate-dextrose (CPD) in polyvinyl chloride containers. Immediately after bleeding, blood was subdivided into two samples: the first was stored at 4 °C under blood bank conditions (stored blood) and the second was used for ageing in protein-free medium (aged erythrocytes). The red cells of the second sample were sedimented by centrifugation at $1100 \times g$ for 15 min. The buffy coat was collected by aspiration and discarded. Complete removal of platelets and leukocytes from the erythrocyte suspensions was obtained by the method of Beutler *et al.* [10]. Packed erythrocytes were suspended in a phosphate-buffered Ringer solution composed of NaCl 140 mM, KCL 3 mM, Na_2HPO_4 8 mM, KH_2PO_4 1 mM, CaCl2 0.9 mM and $MgCl_2$ 0.5 mM. Incubation at 37 °C was carried out for 60 h with gentle shaking to a final hematocrit value, corresponding to the original value, at a pH of 7.2. Although samples were prepared under sterile conditions the incubation mixture contained 200 $\mu g \times ml^{-1}$ Avocin. Sterility tests were performed throughout the experiments and proved to be negative.

At predetermined intervals (0, 17, 35 days for stored blood and 0 and 60 h for aged erythrocytes) aliquots of 2 ml were used for ESR measurements.

Furthermore, at the same time aliquots of 2 ml of stored blood were centrifuged at $3000 \times g$ for 10 min, the plasma was discarded and the red cells (washed erythrocytes) reconstituted in normal saline before the ESR measurements.

ESR measurements

ESR measurements were carried out with an ER 200-SRCB Bruker spectrometer operating at X-band ($\omega_0 = 9,78$ GHz). The external magnetic field H_0 was calibrated with a microwave bridge ER 041 MR Bruker wavemeter and the temperature was controlled with an ER 4111 VT Bruker device (the accuracy was +1 °C). In order to obtain reproducible results, the samples were placed into calibrated quartz capillary tubes permanently positioned in the resonance cavity. The intensity of the X-band ESR signals was obtained from the formula:

$$I = (\Delta H)^2 \times h$$

where ΔH is the linewidth of the mid-field hyperfine line of the spectrum of the MAL-6 and h is the corresponding height. MAL-6 was purchased from Syva and used without further purifications. The MAL-6 labeled samples were prepared by adding 0.1 ml of 1 mM MAL-6 solution to 1 ml of packed red cells. The decrease in the height of the mid-component of the MAL-6 ESR spectrum as a function of time was used in order to carry out kinetic information about MAL-6-blood and MAL-6 red cells interactions. The ESR height of the mid-component was taken as line intensity given the failing in variation of linewidth. Measurement starting point for each sample was 5 min after the addition of the spin-label to the suspension of whole blood and red cells respectively.

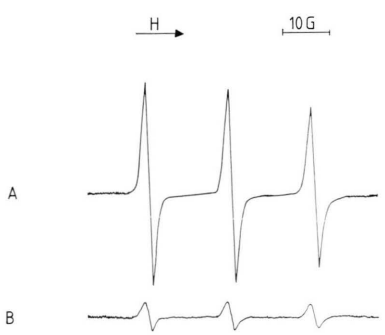
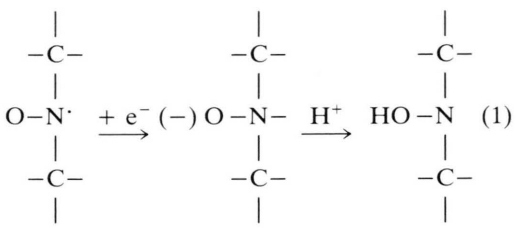


Fig. 1. ESR lineshape of MAL-6 fresh blood system at $t = 0$ (A) and $t = 50$ min (B). [MAL-6] 2×10^{-4} mol $\times$ dm^{-3} $T = 27$ °C.

Results

The kinetic method used in the paper is based on the direct ESR evidence of the rapid redox processes involving the MAL-6 spin label in the presence of human erythrocyte membranes. Assuming a first-order reaction for the MAL-6 reduction to the diamagnetic hydroxylamine, the reaction rate can be easily followed by monitoring the decrease of the ESR signal intensity as a function of time [4]:



(1)

Kinetics of 1 obeys a simple exponential relationship:

$$\frac{-dN}{dt} = K_1 N \qquad (2)$$

$$N = No\ e^{-k1t}$$

where No is the MAL-6 concentration at $t = 0$ min, N is the actual concentration (at the time t) and K_1 is the kinetic constant.

It is known [11] that $N(t)$ is directly related to the $I_{II}(t) = (\Delta H)^2_{II} x h_{II(t)}$, where H_{II} is the linewidth of the second absorption line in the experimental spectrum, h_{II} is the corresponding height and I_{II} is the line intensity.

Under the same experimental conditions, the lineshape of the various nitroxide-erythrocyte samples remains constant for all the measurements, so that the K_1, the kinetic constant, can be directly obtained by plotting $\ln h_{II}$ or $\ln I_{II}$ *vs.* time. The independence of ΔH on time allows us to exclude the presence of effect due to the changes in O_2 concentration of the samples during the ESR measurements.

In the present study we prefer to use intact erythrocytes instead of the ghosts because recent investigations [12] have indicated that significant alterations in protein-protein interactions occur in ghosts with respect to intact red cells.

Fig. 1 shows the comparison between the X-band ESR spectra of the MAL-6 in washed erythrocytes stored under blood bank condition at $t = 0$ and at $t = 50$ min; in this system the ESR measurements were carried out immediately after mixing the MAL-6 solution with the erythrocyte samples. Both the

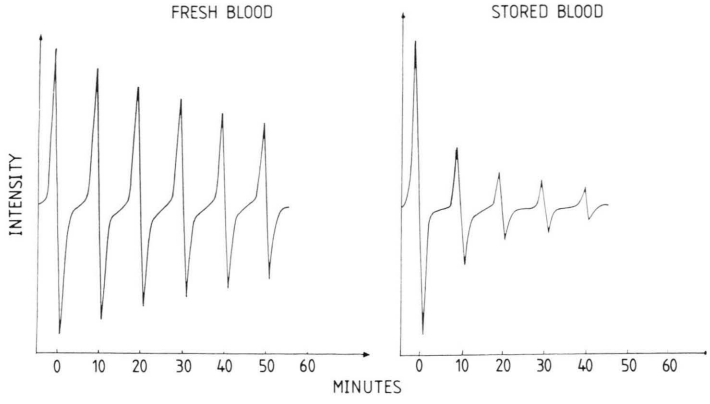


Fig. 2. ESR spectra as function of time expressed as arbitrary units from whole blood treated with MAL-6. Left panel, fresh blood; right panel, stored blood $[MAL\text{-}6] = 2 \times 10^{-4}$ mol $\times$ dm^{-3}.

spectra are typical of "pure" MAL-6 nitroxide aqueous solution; the lineshape excludes any evidence of strongly immobilized spin-label. This fact arises from the used experimental procedure; the features of immobilized ESR signal only appear after an appropriate period of incubation of the erythrocyte ghosts treated with MAL-6 [5]. The intensity decrease must therefore be attributed to redox reaction of the MAL-6 with active components of the red cells; the substantial loss of paramagnetism indicates the rapid reduction to non-paramagnetic derivatives of the spin label.

Fig. 2 shows the comparison between fresh and stored blood in terms of ESR intensity reduction; there is a marked increase of the rate of decomposition of the radical interacting with 35 days stored blood in respect to blood examined at 0 time (fresh blood). Fig. 3 shows the plot of the ln of the MAL-6 (second absorption line intensity) as a function of time relative to the MAL-6 fresh blood system (left side) and to the MAL-6 stored blood system (right side). The slope of the plots directly gives the kinetic constant values k (min^{-1}) of the redox reaction involving the spin label and the two erythrocytic systems. It is evident the noticeable increase of K when the MAL-6 interacts with stored blood.

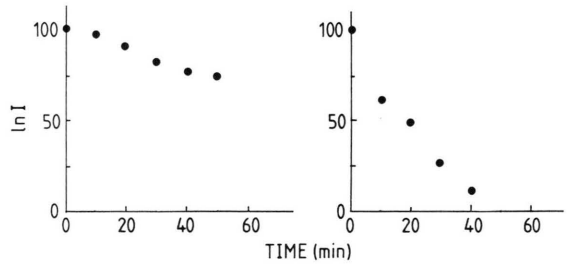


Fig. 3. Plot of ln$I$ (intensity expressed as arbitrary units) *versus* time [min]. Left side, MAL-6-fresh blood system; right side, MAL-6-stored blood system. $[MAL\text{-}6] = 2 \times 10^{-4}$ mol $\times$ dm^{-3}.

The comparison between fresh washed erythrocytes and stored washed erythrocytes in terms of ESR intensity reduction is shown in Fig. 4 by a

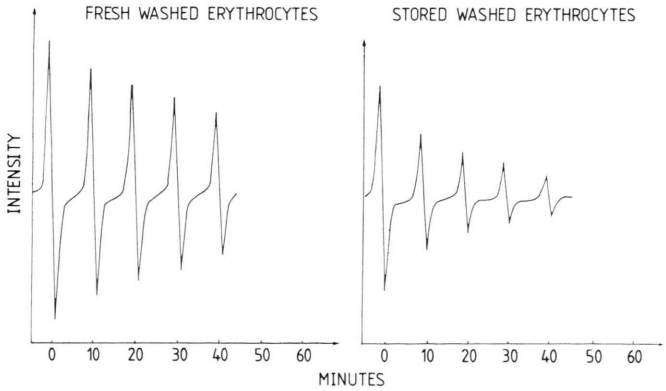


Fig. 4. ESR spectra as function of time expressed as arbitrary units from washed erythrocytes treated with MAL-6. Left panel, fresh washed erythrocytes; right panel stored washed erythrocytes $[MAL\text{-}6] = 2 \times 10^{-4}$ mol $\times$ dm^{-3}.

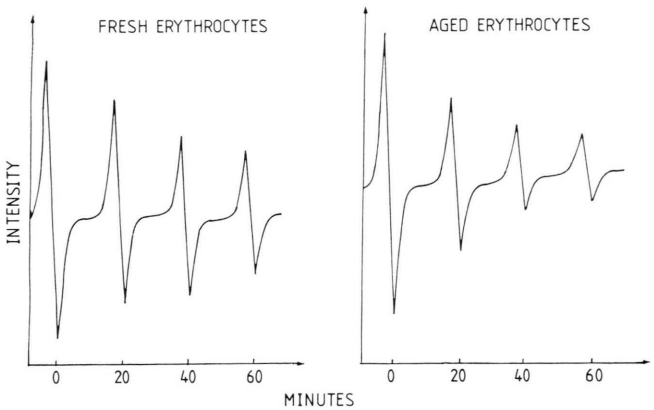


Fig. 5. ESR spectra as function of time expressed as arbitrary units from fresh erythrocytes (left panel) and aged erythrocytes (right panel) treated with MAL-6. [MAL-6] = 2×10^{-4} mol × dm^{-3}.

marked variation of the MAL-6 redox rate. Similar variations can be observed also in erythrocytes incubated at 37 °C (Fig. 5).

Table I shows kinetic values obtained from the corresponding ln$I$ against time (see Fig. 3, as representative example). Kinetic constants for stored blood gradually increase from $1.7 \times 10^{-2} \times \text{min}^{-1}$ to $4.8 \times 10^{-2} \times \text{min}^{-1}$; the same trend is observed for washed erythrocytes. Furthermore, the kinetic constants for aged erythrocytes increase from $1.5 \times 10^{-2} \times \text{min}^{-1}$ to $3.5 \times 10^{-2} \times \text{min}^{-1}$.

Experiments carried out by measuring the ESR spectra of plasma gave kinetic constant of about $3 \times 10^{-3} \times \text{min}^{-1}$.

Table I. Kinetic constants ($K \times 10^{-2} \times \text{min}^{-1}$) expressed as mean ± SD.

Samples	Days of storage at 4 °C		
	0	17	34
Blood	1.7 ± 0.6	3.3 ± 1.2	4.8 ± 2.1
Washed erythrocytes	1.5 ± 0.3	3 ± 0.3	4.6 ± 1.3
	Hours of incubation at 37 °C		
	0	60	
In vitro aged erythrocytes	1.5 ± 0.3	3.5 ± 0.6	

$P < 0.05$. Two tailed T Test for paired samples (n. 5).

Discussion

Our results show a noticeable increase in MAL-6 consumption in stored erythrocytes with respect to fresh erythrocytes and suggest that during storage of blood profound modifications occur in erythrocyte membranes.

The differential consumption of MAL-6 showed by the ESR data is indicative of an increasing susceptibility to oxidative damage of the erythrocytic systems. The experimental evidence strongly suggests that changes in the original membrane structure occur during blood storage or during an artificial ageing of red cells. Previous works have demonstrated that during storage under blood bank conditions [13] or during *in vitro* ageing [14, 15] erythrocytes release vesicles and this may represent one of the major lethal injuries to red cells [8]. The crenation depends on the lipid bilayer and on the spectrin network [16, 17]. Moreover erythrocytes aged in protein-free media undergo a rapid process of ageing with loss of about 50% of sialoglycopeptides from the membrane [18]. The rearrangement of the membrane components was observed also by Bartosz [19] in aged cow erythrocytes; in these cells lipid fluidity decreased and the ESR spectra of membrane-bound MAL-6 evidenced alterations in the state of the membrane proteins. Recently Snyder *et al.* [20] have demonstrated that SH groups on spectrin play a relevant role in the formation of spectrin-hemoglobin complex following an oxidant event. Thus the oxidative damage of the membrane skeleton demonstrated in this work by the redox interaction of the MAL-6 with the network of the erythrocytic membrane components, may provoke the formation of spectrin-hemoglobin complex with subsequent alterations in cell shape and decreased membrane deformability.

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