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The effects of mitomycin-C and temperature on dynamical properties of human erythrocyte membrane

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Abstract

Alterations produced by mitomycin-C (hereafter MC) and temperature changes in human erythrocyte membrane molecular dynamics were studied, in vitro, by spin-labeling ESR technique. Erythrocyte cells were spin-labeled with 5- and 16-doxyl-stearic acids, 5- and 16-DSA, respectively, and membrane fluidity was quantified by measuring the changes in the order parameter (S), correlation time (τ), phase transition temperature and activation energies. Erythrocyte cells were first labeled with 5-DSA and 16-DSA at 37°C for 10 min, then they were treated with MC of three different concentrations (2.5; 5 and 10 μM) for various time (0–300 min) at the same temperature. Experimental results indicate that treatment time and applied MC concentrations do not produce any significant reproducible changes in the spectral parameters. However, change in temperature was observed to alter significantly S and τ parameters that show biphasic character in the temperature range of 5–50°C. Activation energy values of hydrocarbon chains above and below transition temperatures for untreated and MC-treated erythrocyte cells were also determined, and the effect of MC on these energy values were discussed. © 1997 Elsevier Science B.V.

Keywords: Mitomycin-C; Temperature; Human; Erythrocyte; ESR

1. Introduction

Mitomycin-C (hereafter MC) is an antitumor antibiotic first isolated by Harta et al. [1] from the broth of streptomyces caespitosus. By its thermal stability, high melting point, ultraviolet absorbtion peak, solubility in organic solvents, etc., it is distinguished from other mitomycin fractions. Although MC has strong activity against bacteria and viruses, its primary medical use is as an antineoplastic agent in the treatment of various solid tumors such as lung, breast, colon, stomach, pancreas, osteogenic sarcoma, etc. [2–7]. MC is also active against a relatively broad spectrum of experimental tumors, including both haematological and solid types [8–10]. It disappears rapidly from the plasma; there is no evidence of specific tissue localization and one third or less of the injected dose is recovered in the urine [11]. At therapeutic doses in man, MC may produce bone marrow depression with leukopenia, thrombocytopenia, bleeding and increased susceptibility to infection [11]. The other side effects of this drug include nausea, vomiting alopecia, renal, cardio- and pulmonary toxicity. [7,12–14].

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MC has a more profound effect on DNA, at least on DNA in situ. It does not react directly with purified DNA, the antibiotic first must be reduced either enzymatically or chemically to show its effects. In the cells, MC is metabolically reduced to hydroquinone derivatives by a quinone reductase (diaphorase), and these compounds alkylate and extensively crosslink DNA [10,15,16]. The apparently specific attach of mitomycin on the structure and function of DNA in vivo cannot, however, be attributed to absolute specificity for DNA as a substrate for alkylation. MC will also react nearly as well with RNA, and indeed will bind to protein, although less effectively [16,17].

Besides these alkylating and crosslinking actions of MC in vivo, there has been, however, one report in the literature suggesting that MC may have intercalating capabilities in vitro as well [18]. Since then, this last feature of MC is not explored at all, although it is widely used in clinical applications as an antitumor antibiotic agent. Intercalation of MC into plasma membranes and its possible interactions with lipids and proteins would produce changes in the physical properties of these membranes which, in turn, could alter their functions. The aim of the present work is to study in vitro, the possible effects of MC on human erythrocyte membrane physical properties using spin-labeling ESR technique in the temperature range of 0–50°C.

2. Materials and methods

2.1. Membrane model

Human erythrocytes were used as a membrane model system, since the membranes of erythrocytes were luckily the best-understood of all cellular membranes in terms of molecular composition and function [19]. Fresh, healthy human blood, drawn from volunteers, was collected in citrate-phosphate dextrose (CPD) in polyvinyl chloride containers and stored at 4°C for 1–3 h. The blood was then washed with 4°C phosphate-buffered saline (PBS) solution of mM composition NaCl 145, KCl 5, $NaPO_4$ 5 with a pH of 7.4, and then centrifuged at 3500 rpm for 10 min. The buffy coat was carefully collected by aspiration and discarded. This step was repeated at least three successive times. The erythrocytes were then suspended in PBS to an haematocrit of 20%.

2.2. Spin-labeling and treating with mitomycin-C

Fatty acid spin-labels of 5-doxylstearic acid (5-DSA) and 16-doxylstearic acid (16-DSA), which have a stable nitroxide radical ring at the C-5 and C-16, positions (counted from the carboxyl group of the acyl chain), respectively, were used. Stearic acid spin-labels were dissolved in ethanol. To 3 μg of probe, evaporated under nitrogen stream, was added an amount of membranes corresponding to 0.3 mg lipids to keep spin-label concentrations low enough, and therefore to avoid any spin–spin interaction. At room temperature, the time needed for the spin-label incorporation into membranes was very short and, after 5–10 min, the spin-labels were completely associated with the membranes. At the end of spin-labeling step, labeled erythrocytes were washed twice with phosphate-buffered saline solution to remove unincorporated spin-labels.

Spin-labeled erythrocytes of final haematocrit 20% were treated with various concentrations of MC (2.5; 5 and 10 μM) for 10; 25; 50; 100; 150; 200; 250 and 300 min at 37°C in a shaking thermostat bath. After the treatment, the erythrocytes were suspended in 10 volumes of PBS solution at 4°C and centrifugated at $3000 \times g$ for 3 min, and the pellet was sucked into a 50-μl micropet capillary and sealed at both ends with Critoseal. Care was taken to avoid direct contact of the cell suspension with Critoseal and avoid leakage of Mn^{2+} ions from the seal into the cell suspension.

2.3. ESR spectroscopy

Spectra were recorded on a Varian E-line 9 in. spectrometer operated at 9.5 GHz, with TE_{102} cavity resonator, 3300 field set, 100-kHz field modulation, 1.25 G peak to peak modulation amplitude and 10 mW microwave power. The difficulties in determining the intensities of the low-and high-field lines were overcame either by increasing the gain of the spectrometer, or by setting the modulation amplitude at 5 G without apparent changes in the $2T'_{\|}$ values. Sample temperature inside the microwave cavity was monitored with a digital temperature control system

(Bruker ER 4111-VT). The latter gives the opportunity of measuring the temperature with an accuracy of ±0.5°C at the site of the sample. The temperature of the samples was changed between 5–50°C with a 5°C increment. To establish a complete thermal equilibrium, samples were kept for 5 min at each measuring temperature before recording the spectrum.

2.4. Spectral analysis

ESR spectra obtained from membranes of intact human erythrocyte cells with the spin labels 5- or 16-DSA show contributions mostly from spin-labels of restricted motion with negligible contributions from free-moving spin labels. The ESR spectra were evaluated by calculating the motionally averaged nitrogen hyperfine tensor components $2T'_{\parallel}$ and $2T'_{\perp}$, from which S was calculated according to the following equation [20]:

$$S = \frac{T'_{\parallel} - T'_{\perp} - C}{T' + 2T'_{\perp} + 2C} \times \frac{T_{zz} + T_{xx} + T_{yy}}{T_{zz} - 1/2(T_{xx} + T_{yy})} \quad (1)$$

where $T'_{\parallel}$ and T'_{-} are the hyperfine parameters (in gauss) measured directly from experimental spectra and T_{xx}, T_{yy}, T_{zz} are the single crystal hyperfine tensor principal elements of the relevant spin-label. The constant C is a correction factor and is given as

$$C = 1.45\left[1 - \frac{T'_{\parallel} - T'_{-}}{T_{zz} - 1/2(T_{xx} + T_{yy})}\right]$$

Due to the anisotropy of the erythrocyte membrane, the calculated order parameters are not true order parameters, but the apparent order parameter as well as $2T'_{\parallel}$ measurements may be used to obtain information on the dynamic behavior of the membrane. No measurable clustering effect of spin-labels was observed for spin-label/membrane lipid ratio (1/100) adopted in the present work. That is, the values of the parameters were not affected by spin–spin interaction and clustering.

The rotational correlation time (τ), was calculated using the following equation [21]:

$$\tau = 3.418 \times 10^{-10} \times \Delta H(0)\left[\sqrt{\frac{h(0)}{h(-1)}} - \sqrt{\frac{h(0)}{h(1)}}\right] \quad (2)$$

where $h_{(0)}$, $h_{(1)}$ and $h_{(-1)}$ are the peak height of the center, low-field and high-field lines, respectively, and $\Delta H(0)$ is the width of the central line. Eq. (2) is suitable for a rod-like molecule and especially for perpendicular resonance spectra [22]. Because of assumptions made in the derivation of Eq. (2), its validity is questionable for rotational correlation times longer than about 2 ns.

For a rod-like molecule of radius r, and length l, the rotational correlation time is related to the rotational viscosity as [22]:

$$\tau = \frac{2\pi l \eta r^2}{kT} \quad (3)$$

where η is the viscosity coefficient, T is the absolute temperature and k is the Boltzmann constant. On the other hand, the activation energy E_{ac} of rotational viscosity is given by Andrade's equation [23] for viscosity, that is,

$$\eta = B\exp[E_{ac}/RT] \quad (4)$$

where R and T are the gas constant and absolute temperature, respectively. From the free volume theory, B may be given by [24].

$$B = B_0 T^{3/2} \quad (5)$$

Using above equations, we have constructed Andrade plots for the activation energies of microviscosity of the labeled erythrocyte membranes untreated and treated with MC.

2.5. Chemicals

5-DSA and 16-DSA stearic acid spin-labels were purchased from the Sigma Chemical (St. Louis, Mo, USA). MC and all other chemicals used for sample preparation and treatment were of reagent grade from Fluka (Bushs, Switzerland).

2.6. Statistics

Experiments were performed 6–10 times, and standard deviations were calculated. All order parameters and correlation times measured are reported as mean ± S.D. of the number of experiments performed. The S.D. values were determined using paired or non-paired Student's t-test depending on appropriateness [25]. P values of less then 0.05 were considered significant changes. The phase transition temperatures and activation energies were determined using the linear regression analysis 'breakpoints' computer program [26].

3. Results

The effect of spin-labeling time on spectral parameters was determined first, to adopt an appropriate ultimate labeling time. To achieve this goal, time evolution of ESR spectra were studied by changing spin-labeling time from 5 to 100 min and recording the spectra at the end of each labeling period for erythrocyte cells kept at 37°C. Measurements were shown that for labeling time longer than 10 min, neither the shape of the spectra nor the spectral parameters (S, τ, E_{ac}) of 5- and 16-DSA were changed [27] within experimental error limits.

A slight intensity change up to a labeling time of 15 min was observed. This shows that some spin-labels still existing in buffer solution would incorporate into the erythrocyte membrane up to 15 min labeling time. Therefore, a labeling time of 10 min was adopted for the rest of the present work. ESR spectra of 16-DSA were found to be considerably different from the spectra of 5-DSA. Due to the fact that the motion of 16-DSA was nearly isotropic, only one minimum at high magnetic field was found to exist in the spectra of this label. Therefore, $2T'_{\|}$ was calculated from the difference between this minimum and the maximum at low field.

3.1. Effect of mitomycin-C

ESR spectra analysis of stearic acid spin-labels incorporated into erythrocyte cells treated with different drugs can provide an evaluation of the effects of the latter on the motional freedom of the inserted spin labels. Information on the dynamical behavior of spin-labels was obtained by outer hyperfine splittings ($2T'_{\|}$), S and rotational correlation time (τ) calculated from Eqs. (1) and (2), respectively. Before making a detailed analysis of ESR spectra, we wanted to be sure of the effects of treatment time with MC on spectral parameters. Adopting a sublytic drug

Table 1
The effect of treatment time on spectral parameters of 16-DSA spin-label inserted into erythrocyte cells treated with 2.5 μM MC at 37°C

Parameters	Treatment time (min)							
	10	25	50	100	150	200	250	300
S	0.186 (0.007)	0.196 (0.006)	0.192 (0.007)	0.191 (0.005)	0.190 (0.004)	0.187 (0.005)	0.194 (0.005)	0.196 (0.007)
$\tau \times 10^{10}$ (s)	6.2 (0.5)	5.8 (0.4)	5.6 (0.4)	5.6 (0.3)	6.3 (0.5)	6.3 (0.5)	6.6 (0.6)	6.2 (0.4)
η (cp)	1.6 (0.2)	1.5 (0.2)	1.4 (0.3)	1.4 (0.3)	1.6 (0.2)	1.6 (0.2)	1.7 (0.3)	1.6 (0.2)

Parameter values were calculated from ESR spectra recorded at 37°C of 5–10 different samples. Figures in parentheses are standard deviations.

Table 2
The effect of MC concentration on spectral parameters of 5- and 16-DSA spin-labels inserted into erythrocyte cells treated with 0, 2.5, 5 and 10 μM MC at 37°C

Spin-labels	Parameters	Concentrations (μM)			
		0	2.5	5	10
5-DSA	S	0.685 (0.006)	0.680 (0.007)	0.684 (0.006)	0.686 (0.005)
	$\tau \times 10^{10}$ (s)	60 (2)	60 (2)	59 (1)	61 (1)
	η (cp)	14.9 (0.5)	15.5 (0.6)	15.7 (0.7)	15.1 (0.5)
16-DSA	S	0.198 (0.005)	0.208 (0.007)	0.205 (0.006)	0.207 (0.005)
	$\tau \times 10^{10}$ (s)	4.8 (0.2)	5.0 (0.3)	5.2 (0.3)	5.1 (0.2)
	η (cp)	1.2 (0.1)	1.3 (0.2)	1.3 (0.1)	1.1 (0.2)

Parameter values were calculated from ESR spectra recorded at 25°C of 6–10 different samples. Figures in parentheses are standard deviations.

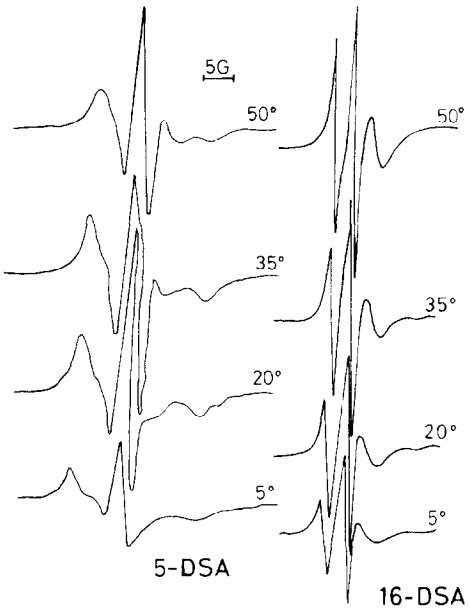

Fig. 1. Spectra recorded at various temperatures for 5- or 16-DSA labeled erythrocyte cells treated with 2.5 μM MC.

concentration (2.5 μM), treatment time was changed from 10 to 300 min keeping the samples at 37°C for 5- and 16-DSA spin labels. Experimental results were shown that, within experimental error limits, treatment time had no significant effect on spectral parameters. Therefore, for the rest of present work, a treatment time of 30 min was adopted for both 5- and 16-DSA inserted erythrocyte cells. To save place, only the results relative to 16-DSA are given here (Table 1).

The effects of drug concentration were also studied staying nearly in situ blood MC concentration limits encountered in patients treated with this drug. 5- or 16-DSA labeled erythrocyte cells were treated with MC of 2.5; 5 and 10 μM concentrations at 37°C for 30 min. For each drug concentration, 6–10 samples were used to get mean parameter values. The results obtained from the analysis of ESR spectra recorded at 25°C are given in Table 2. As seen from this table, in the 0–10 μM concentration range,

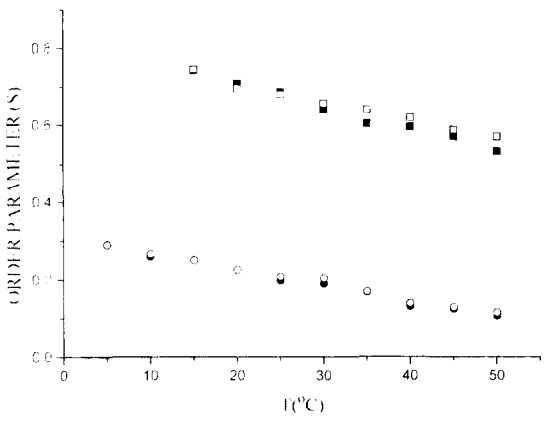

Fig. 2. Variation of order parameter with temperature for untreated and MC-treated erythrocyte cells. (□) 5-DSA, (■) 5-DSA + 2.5 μM MC, (○) 16-DSA, and (●) 16-DSA + 2.5 μM MC.

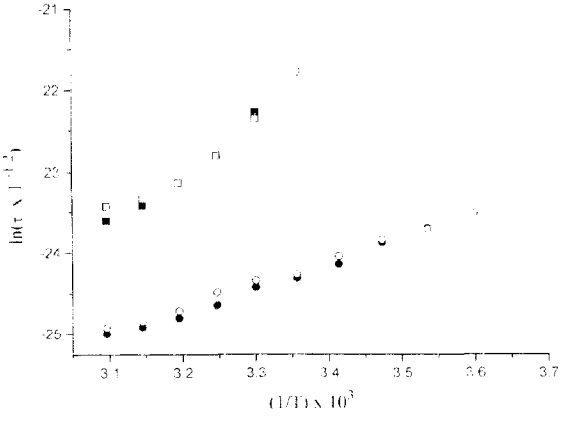

Fig. 3. Andrade plots for rotational correlation times of untreated and treated erythrocyte cell. (□) 5-DSA, (■) 5-DSA + 2.5 μM MC, (○) 16-DSA and (●) 16-DSA + 2.5 μM MC.

Table 3
Activation energies and thermotropic phase transition temperatures for control and 2.5 μM MC treated erythrocyte cells

Spin-label	Type of sample	Activation energy (kcal/mol)		
		Low temperature	High temperature	Break point (°C)
5-DSA	Untreated	18.0 (±1.0)	10.4 (±1.0)	36 (±1)
	Treated	18.3 (±1.0)	5.8 (±0.9)	37 (±1)
16-DSA	Untreated	6.1 (±0.9)	3.7 (±0.8)	40 (±1)
	Treated	5.5 (±0.8)	2.9 (±0.8)	43 (±1)

the treatment of erythrocyte cells with MC in vitro does not create any significant changes in dynamical parameters of spin-labels inserted in the erythrocyte cell membrane.

The order parameter, rotational correlation time and viscosity coefficient near polar head group were large (0.685; 60×10^{-10} s; 14.9 cp, respectively) whereas on moving from the surface into the membrane core, the parameters decreased to 0.198; 4.8×10^{-10} s; 1.2 cp, respectively.

3.2. Effect of temperature

It is well known that temperature affects greatly the behavior of model and biological membranes. In previous studies [28–30], it has been observed that in the 0–50°C temperature range, the erythrocyte membrane presents discontinuous changes in the freedom of motion of nitroxide spin-labels that may be indicative of protein-dependent structural transitions. Therefore, by changing the temperature of the erythrocyte samples from 5 to 50°C and recording the spectra with an increment of 5°C, we have investigated the effect of temperature on the structural features of untreated and MC treated erythrocyte membrane. Some spectra recorded at different temperatures for erythrocyte cells labeled with 5-or 16-DSA spin label and treated with 2.5 μM MC are given in Fig. 1.

From recorded spectra, S and τ were determined for untreated (control) and MC-treated erythrocyte membrane using Eqs. (1) and (2). The data obtained are presented as plots in Figs. 2 and 3. The magnitude of the changes in the measured S and τ values for untreated and MC-treated cells were of the same order as those found by many investigators who have compared membranes of biologically altered cells [31–33]. Both order parameter and correlation time decrease rapidly with increasing measuring temperature for control and MC-treated erythrocyte membrane as seen from Figs. 2 and 3 in the temperature range of 5–50°C. Andrade plots of untreated and treated erythrocyte membranes have been observed to be composed of two approximately straight lines with a break point. The slope of the straight line at low temperature was more steep (gel phase) than that at high temperature (liquid crystalline phase). Discontinuities around break-points were not specific for spin-label or the empirical parameter chosen for spectral evolution. Similar high temperature thermotropic transitions were also observed in other works [28,30,34] for erythrocyte membrane labeled with 5- and 16-DSA spin labels.

3.3. Calculation of activation energies and phase transition temperatures

The activation energies and thermotropic transition temperatures were calculated using the linear regression analysis 'break-points' computer program based on Eqs. (3)–(5) by taking $l = 22.63$ Å and $r = 1.08$ Å. The slopes of linear curves best-fitting the experimental data in the low and high temperature regions were used to calculate activation energies below and above transition temperatures. As has been known, the slopes of these linear curves are related to the activation energies of micro-viscosity of the studied membranes. Calculated activation energy values and thermotropic transition temperatures are given in Table 3. The average activation energies were about 5–18 kcal/mol for gel phase and 3–10 kcal/mol for liquid crystalline phase.

4. Discussion

The analysis of motion of spin-labels in the model and biological membranes is a sensitive and reliable indicator of the physical state of these membranes.

ESR studies give mainly the rotational correlation time and order parameter which, generally, represent the degree of long-range alignments of hydrocarbon chains along the membrane normal, but it is not directly related to rotational correlation time [20]. Nevertheless, the correlation time should be related to short-range alignments of hydrocarbon chains, because in increasing the short range alignments, the distances among hydrocarbon chains are reduced, that is, chain–chain interactions are increased; thus, the rotational correlation time should be increased.

Spin-labeling of erythrocyte membrane with 5- or 16-DSA at 37°C was shown to be completed in great extent in a time interval of 10 min after addition of spin-label molecules to erythrocyte cell samples. Furthermore, changing the treatment time with MC in the range of 0–300 min has been observed to be not producing any significant effect on the shape of ESR spectra and on the spectral parameters of 5- and 16-DSA spin-labels incorporated into erythrocyte cell membrane.

As expected, a big difference in the values of order parameters of 5- and 16-DSA was calculated in the present work for untreated and MC-treated erythrocyte cells in the temperature range of 5–50°C. This is due to the fact that in the core of the membrane, where the doxyl group of 16-DSA penetrates, there exists a more fluid environment than that closer to the membrane surface where the doxyl group of 5-DSA probes. It is well established in the literature that in biological membranes, the rotational correlation time and order parameter, generally, decrease gradually as the depth of doxyl group from the polar surface of membrane increases.

MC concentrations up to 10 μM were observed not having any measurable effect on order parameters and correlation times of the spin-labels used in the present work (Table 2). This is possibly due to the fact that MC was not able to incorporate into the membrane that would modulate lipid–lipid and lipid–protein interactions by producing changes between chain–chain and chain–protein distances in the membrane. Furthermore, the results given in Table 2 indicate that interaction between lipid polar head groups, which determine, to a large extent, segmental chain motions at the surface of membrane, and MC molecules, is very weak so that it cannot produce any measurable changes in experimentally determined spectral parameters. This conclusion is in accordance with the observation that MC should undergo chemical and enzymic reduction before its alkylating effect appears [10]. Activated MC may perturb membrane lipid structure; that is, it could create changes in the apparent order parameter, through two possible mechanism: (1) a direct interaction of drug with membrane protein reflected in the lipid bilayer; and (2) a perturbation of lipid structure that leads to perturbation of proteins via lipid–protein interaction.

Calculated spectral parameters S and τ were observed to vary with temperature, as expected. These variations are shown in Figs. 2 and 3 for untreated and MC-treated erythrocyte cells. The magnitude of the changes in the measured S- and τ values for untreated and MC-treated cells were of the same order as those found in the literature for erythrocyte membrane [28,30,35]. Since, 5- and 16-DSA spin-labels are composed of very flexible hydrocarbon chains, it seems unreasonable to assume that these spin-labels can be treated as rigid rodlike molecules. However, without obtaining the rotational viscosity, the activation energy of rotational viscosity was easily calculated from Andrade plots, which showed the existence of phase transition of hydrocarbon chain regions. From Figs. 2 and 3, it is clear that changes in temperature have a greater effect on erythrocyte membrane in the hydrophobic core of the membrane compared with the membrane surface. This result is in agreement with those reported in the literature [28,35,36].

Erythrocyte membrane has been reported to undergo more than one thermotropic phase transitions using ESR and spin-labeled fatty acid labels [28,30,34]. In the present work, low temperature phase transition was not observed due to the fact that in the temperature range of 0–20°C, proper determinations of experimentally measured quantities ($T'_{\parallel}$, $T'_{\perp}$, $h_{(1)}$, $h_{(0)}$, $h_{(-1)}$, $\Delta H(0)$) which were used in Eqs. (1) and (2) to calculate S- and τ parameters, were not possible. Furthermore, in the temperature range of 0–20°C, the validity of the assumptions made in deriving Eqs. (1) and (2) is questionable.

5-DSA spin-label monitors the relatively ordered membrane regions close to the surface, while 16-DSA monitors the region in the core of membrane. From structural standpoint, below the transition tempera-

ture the hydrocarbon chains in the membrane are relatively rigid all-*trans* conformation. As the temperature is increased up to the transition point, the hydrocarbon chains disorder by undergoing rapid *trans*-gauch rotational isomerizations along the chains [36]. The results of the present and similar works reported in the literature suggest that the spectrin–actin network and proteins that link the skeletal network to the membrane are important in mediating this *trans*-gauch rotational isomerization along the hydrocarbon chains, and that protein unfolding could be at the origin of this rotational isomerization [28,36]. Our experimental results show that this rotational isomerization occurs at about 36°C (monitored by 5-DSA) in the polar region of membrane, while in the core, this transition occurs at about 40°C (monitored by 16-DSA), and that while the treatment with MC does not create any alteration in the thermal transition undergoing at about 36°C, it causes an increase of about 3°C in the transition temperature detected by 16-DSA.

5. Conclusion

Our results indicate that in vitro: (a) up to a concentration limit of 10 μM, MC does not produce any measurable alteration in erythrocyte membrane dynamic features at body temperature; (b) the use of the 5- and 16-DSA spin-labels to study thermotropic properties of erythrocyte membranes provides a way to identify thermotropic breaks that can be selectively modified by specific protein extraction. Thus, as far as the concentration is kept below 10 μM, treatment with MC cannot create any dynamical and, therefore, any functional changes in the erythrocyte membrane of the patients, unless any in situ chemical and/or enzymic reduction of MC would occur.

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