

***In Vitro* Effects of Colchicine on Human Erythrocyte Membranes: An ESR Study**

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Colchicine (CC) is an antimitotic and antitubular agent which is currently used in medicine as a drug to treat various diseases. In spite of its widespread use the interaction of this drug with membranes is not yet known clearly. To characterize alterations in membrane molecular dynamics produced by CC, human erythrocytes were spin-labeled with 5-doxydstearic acid (5-DSA) and 16-doxydstearic acid (16-DSA), and membrane fluidity was quantified by measuring the changes in the order parameter (S) which reflects the anisotropy due to restricted motional averaging, correlation time (τ_c) representing the spin label tumbling rate in the sample and phase transitions derived from ESR measurements. CC induced significant changes in both order parameter and correlation time at the surface of 5-DSA labeled membrane, but this was not the case at the core of 16-DSA labeled membrane. Order parameter, S and rotational correlation time, τ_c , showed a biphasic character in the temperature range of 5–50 °C. This fact was used to calculate phase transition temperatures and activation energies of untreated (control) and treated erythrocyte cells with CC.

Introduction

Colchicine (CC) is a antimitotic and antitubular agent which is currently used in medicine as a drug in treating various diseases such as acute gout, hepatitis and pulmonary fibrosis, familial Mediterranean fever, cirrhosis, Behçet's syndrom etc (Jolobe, 1994; Mourella and Meiza, 1989; Peters *et al.*, 1993; Benchetrit *et al.*, 1994; Mourella *et al.*, 1981; de Bois *et al.*, 1991). In toxic doses, CC causes metaphase arrest due to disruption of the normal functioning of the mitotic spindle. Spindle function is disrupted due to interference with microtubule polymerization (Sackett and Varma, 1993). This interference is mediated by binding of CC to the microtubule subunit protein tubulin which is a heterodimer composed of two similar but non-identical subunits, α and β tubulin. Under most conditions, the tubulin-CC complex does not polymerize, but can nonetheless add to the growing end of a microtubule. CC binding prevents the formation of an intra- β -chain cross-link between reactive sulfhydryls indicating a shift intubulin concentration.

Considerable evidence has accumulated indicating that microtubules are able to modulate functions of the plasma membrane including hormone responsiveness (Hegmann and Fishman, 1980) capping of cell surface molecules (Malawista *et al.*, 1978), and endo- and exocytosis (Wehland *et al.*, 1982). Early studies have demonstrated effects of microtubule depolymerizing agents on phagocytosis-induced alterations in membrane viscosity (Berlin and Fera, 1977). These observations suggested us to carry out an investigation on the effects of CC on fluidity and thermotropic phase transitions of human erythrocyte membrane.

Although the occurrence of structural transitions in plasma membranes of mammalian cells has been described by several groups (Inesi *et al.*, 1973; Whetton *et al.*, 1983; Forte *et al.*, 1985), structural changes involved in these transitions are not well understood due to the high level of complexity reflected in a high level of anisotropy of these membranes. Structural transitions in the 0–50 °C temperature range of the erythrocyte cell membrane has been reported using a variety of physicochemical techniques (Nigg and Cherry, 1979; Galla and Luisetti, 1980; Minetti *et al.*, 1984) but structural changes involved in these transitions are still unclear. However, noteworthy is the lateral mobility of glycoproteins which changes dis-

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continuously with temperature (Forte *et al.*, 1985; Nigg and Cherry, 1979) and appears to be controlled by skeletal proteins (Golan and Weatch, 1980). Temperature affects not only membrane enzymatic activities but also resealing and hemolysis processes.

Spin label electron spin resonance (ESR) spectroscopy is a useful tool to study reporter group sensitivity to the mobility and chemical environment of its host molecules (Berliner, 1976; Lai, 1986). In this study, motional freedom of fatty acid spin labels (5-DSA and 16-DSA) inserted into the membrane of healthy human erythrocyte cells treated with different doses of CC has been used as a measure of membrane fluidity on the surface and in the core of membrane to determine the effects of CC and temperature on dynamic and structural features of erythrocyte membrane. Alterations in motional freedom of the spin labels are reflected as changes in the order parameter, S , rotational correlation time, τ_c , and phase transition temperature, T_c , which can be calculated from ESR measurements. An S value equal to one represents no motional freedom of the spin label, as would be the case in a crystal lattice, whereas S values less than one represent increasing motion of the spin label which can be interpreted as increased membrane fluidity.

Membrane fluidity is a function of many variables, including the nature of the membrane kinds (type of phospholipid, fatty acid chain length and degree of unsaturation, head group and cholesterol content), temperature, water content, presence of divalent cations and the complexity of protein-lipid interactions. Our results support a model in which microtubules interact directly or indirectly with the cell membrane and alter membrane function by changing the motional freedom of the membrane lipids.

Materials and Methods

Membrane model

Human erythrocytes were used as a membrane model system, since the membranes of erythrocytes are the best-understood of all cellular membranes in terms of molecular composition and function (Chasis and Shohet, 1987). Fresh healthy human blood, drawn from volunteers was collected in citratephosphate dextrose (CPD) in poly-

vinyl 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, NaPO₄ 5 with a pH of 7.4, and then centrifuged at 1500 ×g for 10 min. The supernatant was carefully collected by aspiration and discarded. This step was repeated at least three successive times. The erythrocytes were then suspended in PBS so that the volume percentage of erythrocyte be 20%.

Spin-Labeling and treating with colchicine

Fatty acid spin-labels of 5-doxylosteoric acid (5-DSA) and 16-doxylosteoric acid (16-DSA), which have a stable nitroxide radical ring at C-5 and C-16, positions (counted from the carboxyl group of the acyl chain), were used, respectively. Stearic acid spin-labels were dissolved in ethanol. To 3 µg of probe, evaporated under nitrogen stream, an amount of membranes was added corresponding to 0.3 mg lipids to keep the 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, erythrocytes were washed twice with phosphate-buffered saline solution to remove unincorporated spin-labels.

Spin labeled erythrocytes were treated with various concentrations of CC (5, 10 and 20 µM) for 5, 10, 20, 30, 45, 60 and 90 min at 37 °C in a shaking thermostat bath. These concentrations were sufficient to cause complete depolymerization of microtubules in human erythrocyte cells. After the treatment, the erythrocytes were suspended in 10 volumes of PBS solution at 4 °C and centrifuged at 3000 ×g for 3 min, and the pellet was sucked into a 50 µl capillary and sealed at both ends with sealing wax. Care was taken to avoid direct contact of the cell suspension with sealing wax so as to avoid leakage of Mn²⁺ ions from the seal into the cell suspension.

ESR spectroscopy

Spectra were recorded on a Varian E-line 9" spectrometer operated at 9.5 GHz, with TE₁₀₂ cavity resonator, 3300 field set, 100-kHz field modula-

tion, 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 overcome either by increasing the gain of the spectrometer or by setting the modulation amplitude at 5 G without apparent changes in the $2T'_{\parallel}$ 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 $\pm 0.5^{\circ}\text{C}$ at the site of sample. The temperature of the samples were changed between $5\text{--}50^{\circ}\text{C}$ with a 5°C increment. To establish a complete thermal equilibrium, samples were kept for five minutes at each measuring temperature before recording the spectrum.

Spectral analysis

ESR spectra obtained from membranes of intact human erythrocyte cells with the spin labels 5-DSA and 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 the order parameter, S , were calculated according to the following equation (Berliner, 1976).

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

where T'_{\parallel} and T'_{\perp} are the hyperfine parameters (in gauss) measured directly from experimental spectra as shown in Fig. 1 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

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

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 mass 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_c , was calculated using the following equation (Hemminga, 1975).

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

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. Eqn. (2) is suitable for a rod-like molecule and especially for perpendicular resonance spectra (Hyono *et al.*, 1980). Because of assumptions made in the derivation of Eqn. (2) its validity is questionable for rotational correlation times longer than about 2 nsec.

For a rod-like molecule of radius, r , and length, l , the rotational correlation time is related to the rotational viscosity as (Hyono *et al.*, 1980):

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

where η is the viscosity, T is the absolute temperature and k is the Boltzmann constant. On the other hand, the activation energy E of rotational viscosity is given by Andrade's equation for viscosity, that is,

$$\eta = B \exp [E/RT] \quad (4)$$

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

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

Here, B_0 is a coefficient which depends on molar volume. Using above equations, we have constructed Andrade plots for the activation energies of microviscosity of the labeled erythrocyte membranes untreated and treated with CC.

Chemicals

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

Statistics

Experiments were performed four to seven times and standard deviations were calculated. All order parameters and correlation times measured are reported as mean \pm 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 (Dixon and Massey, 1969). *P* values of less than 0.05 were considered significant changes. The phase transition temperatures and activation energies were determined using the linear regression analysis "break points" computer program (Jones and Molitoris, 1984).

Results

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. An experimental spectrum recorded at 37 °C for erythrocytes cells labeled with 5-DSA spin label for 10 min, is shown in Fig. 1. 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 and $2T'_{\parallel}$ was obtained from the difference between this minimum and the maximum at low field.

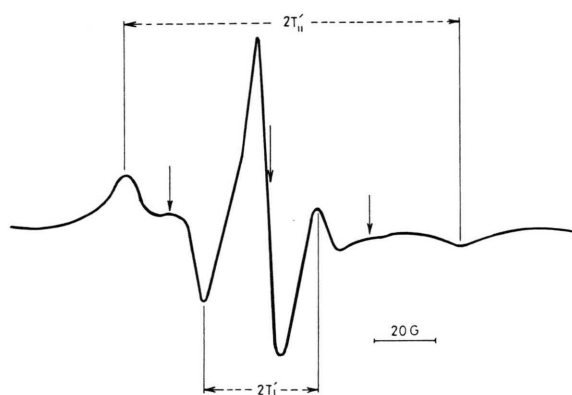


Fig. 1. Typical ESR spectrum of 5-doxylstearic acid (DSA) labeled erythrocyte cells at 37 °C. The arrows indicate the position of freely-moving spin label.

Effect of colchicine

The order parameter and correlation time near polar head groups in the untreated membrane were large (0.601 and 19.1×10^{-10} s) whereas on moving from the surface into the membrane core, *S* and τ_c decreased to 0.150 and 3.30×10^{-10} s, respectively (Table I). This is partly due to the fact that in the intact erythrocyte membrane, the lipids distribute asymmetrically in double leaflets, inducing heterogeneity in the fluidity; a more rigid outer layer and a more fluid inner layer.

Table I. Variation of order parameter (*S*) and correlation time (τ_c) with CC concentration at 37 °C for 5- and 16-doxyl stearic acid (5-DSA and 16-DSA) labeled erythrocyte cells.

Spin-label	Colchicine (CC) concentration [μ M]	Order parameter (<i>S</i>)	Correlation time (τ_c) ($\times 10^{10}$ s)
5-doxyl stearic acid	untreated	$0.601(\pm 0.003)$	$19.1(\pm 0.1)$
	5	$0.597(\pm 0.002)$	$18.9(\pm 0.1)^{**}$
	10	$0.593(\pm 0.003)^{***}$	$18.7(\pm 0.2)^{***}$
	20	$0.589(\pm 0.002)^{***}$	$18.5(\pm 0.1)^{***}$
16-doxyl stearic acid	untreated	$0.150(\pm 0.003)$	$3.30(\pm 0.05)$
	5	$0.146(\pm 0.003)$	$3.21(\pm 0.04)^{**}$
	10	$0.143(\pm 0.004)^*$	$3.15(\pm 0.05)^{**}$
	20	$0.141(\pm 0.003)^{***}$	$3.10(\pm 0.03)^{***}$

* $p < 0.02$; ** $p < 0.01$; *** $p < 0.001$.

Three different sublytic concentrations of CC (5, 10 and 20μ M) which were sufficient to cause complete depolymerization of microtubules in human erythrocyte membrane were tried by keeping the labeled erythrocyte cells at 37 °C. The evaluation of the ESR spectra with CC treatment time of spin-labeled erythrocyte cells, was studied first, and a treatment time of 60 min was found to be appropriate to study the effects of drug on membrane properties in the concentration limits of CC given above. From the spectra recorded at 37 °C for treated erythrocyte cells we have determined the values of order parameter, *S*, and correlation time, τ_c , for CC concentrations of 5, 10 and 20μ M using Eqns. (1) and (2). The results obtained for human erythrocyte cells labeled with 5-DSA and 16-DSA are given in Table I. Treatment with CC produces detectable decreases in both correlation times and order parameters of spin-labels (5-DSA and 16-DSA) for applied concentration and this effect increases with increasing CC concentration. The rotational correlation time and order param-

ter also decrease gradually for treated cells as the depth of spin-labels from the polar surface of membrane increases as in the case of untreated cells.

The values of S and τ_c given in Table I are the means of four to seven different measurements using different samples of untreated and treated cells. Figures in parentheses are the standard errors. Mean values and standard errors of similar magnitude were calculated by Korkmaz (1995) and Minetti *et al.*, (1987) from the data which were also obtained with 9 GHz ESR measurements on erythrocyte membrane. The order parameters of 20 μM CC treated cells were found to decreased by $(2.0 \pm 0.3) \%$ and $(6.0 \pm 0.5) \%$ for 5-DSA and 16-DSA, respectively, in comparison to that of untreated cells. This suggested that the effects of CC were less evident with the increase of flexibility gradient toward the surface of the bilayer.

Effect of temperature

Some spectra recorded at different temperatures for erythrocyte cells labeled with 5-DSA or 16-DSA spin label and treated with 10 μM CC are given in Fig. 2. From recorded spectra, order

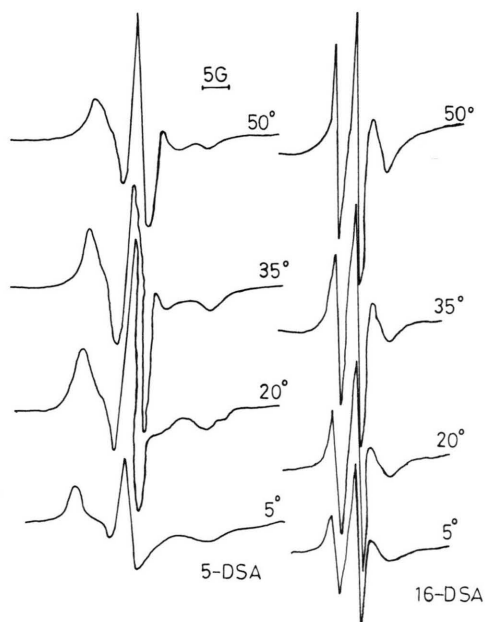


Fig. 2. Spectra recorded at various temperature for 5-DSA and 16-DSA labeled erythrocyte cells treated with 10 μM CC.

parameter, S , and correlation time, τ_c , were determined for untreated (control) and CC-treated erythrocyte membrane using Eqns. (1) and (2). The data obtained are presented as plots in Figs. 3 and 4. The magnitude of the changes in the measured S and τ_c values for untreated and CC-treated cells were of the same order as those found by many investigators who have compared membranes of biologically altered cells (Aszalos *et al.*, 1984; Esser and Russel, 1979; Lai *et al.*, 1980). Both order parameter and correlation time decreased rapidly with increasing measuring temperature for control and CC-treated erythrocyte

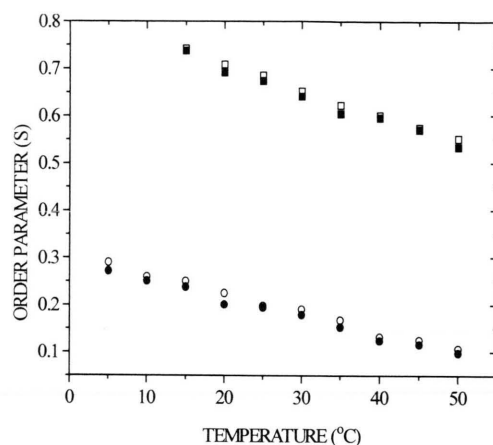


Fig. 3. Variations of order parameters with temperature for untreated and treated erythrocyte cells. \square (5-DSA), \blacksquare (5-DSA + 10 μM CC); \circ (16-DSA) and \bullet (16-DSA + 10 μM CC).

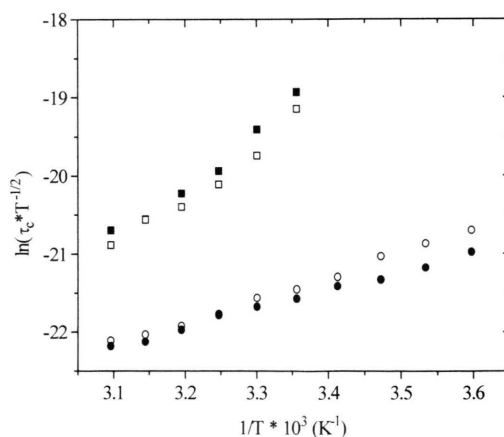


Fig. 4. Andrade plots for correlation times of untreated and treated erythrocyte cells. \square (5-DSA), \blacksquare (5-DSA + 10 μM CC); \circ (16-DSA) and \bullet (16-DSA + 10 μM CC).

membrane as seen from Figs. 3 and 4, in the temperature range of 5–50 °C. This means that increases in temperature and CC concentration have similar effects on the freedom of motion of spin labels, although their magnitudes are different. Andrade plots of untreated and treated erythrocyte membranes have been all observed to be composed of two approximately straight lines with a break point. The straight line at lower temperature was steep (gel phase) and the one at higher temperature had a gentle slope (liquid crystalline phase). Discontinuities around break points were not specific for the spin label or the empirical parameter chosen for spectral parameter evolution. Similar high temperature thermotropic transitions were also observed in other works (Minetti and Di Stasi, 1987; Forte *et al.*, 1985; Wood *et al.*, 1987) for erythrocyte membrane labeled with 5-DSA and 16-DSA spin labels.

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 Eqns. (3), (4) and (5) by taking the length (l) and radius (r) of the molecules as 22.63 Å and 1.08 Å, respectively. 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 is 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 II. The average activation energies were

about 20–75 kJ/mol for gel phase and 12–42 kJ/mol for liquid crystalline phase.

Discussion

A relatively big difference in the values of order parameters, S , for 5-DSA (0.601 ± 0.003) and for 16-DSA (0.150 ± 0.003) in untreated erythrocyte cells at 37 °C was calculated in the present work. This difference is expected because it was shown in the literature that deeper in the hydrocarbon region, where the nitrogen group of 16-DSA penetrates, exists a more fluid environment than compared to that of the membrane surface where the nitroxide group of 5-DSA probes (Berliner, 1976; Aszalos *et al.*, 1984; Mc Farland and Mc Connell, 1971). Similarly, an increase in the motional freedom of spin probes was reported in the literature (Minetti and Di Stasi, 1987; Wood *et al.*, 1987; Cooper *et al.*, 1992) for spin labels probing at the same membrane depths as in our study on erythrocyte membranes. In biological membranes the rotational correlation time and order parameter, generally, decreases gradually as the depth of spin label from the polar surface of membrane increases.

Biological and pharmacological properties of CC vary qualitatively as a function of its concentration. The present work has been focused on the effects of CC in the 5–20 µM concentration range where this drug produced hemolysis protection or “pre-lytic” phenomena in erythrocyte cell. At these concentrations; CC treated erythrocytes showed, although of small magnitudes, a dose-dependent increase (Table I) in the freedom of motion of 5-DSA and 16-DSA spin labels as it was observed by Aszalos *et al.* (1985) on 5-DSA labeled Chinese hamster ovary cells induced by CC and similar antitubular drugs. The dose response results of erythrocyte cells to CC given in Table I, show that changes in ESR spectral parameters are related to the ability of the drug to depolymerize microtubules. As seen from Table I effects of CC on S are observed only with concentrations of CC that detectably depolymerize microtubules. The observed increases with increasing CC concentration at 37 °C in the motional freedom of spin labels can be seen as a result of local unfolding of β -tubulin near the carboxyl end of the protein by CC binding (Sackett and Varma, 1993). This point

Table II. Activation energies and thermotropic phase transition temperatures for untreated and 10 µM CC treated erythrocyte cells.

Spin-label	Type of sample	Activation energy (E) [kJ/mol]		Break point [°K]
		Low temp.	High temp.	
5-doxyl stearic acid	untreated	75.4(±4.2)	43.5(±4.2)	36(±1)
	treated	74.1(±4.2)	37.3(±3.8)	35(±1)
16-doxyl stearic acid	untreated	25.5(±3.8)	15.5(±3.3)	40(±1)
	treated	19.7(±3.8)	15.9(±3.3)	43(±1)

is justified by Aszalos *et al.* (1985) in their work on Chinese hamster ovary cell plasma membrane by immunofluorescence assays. Structural changes occurring in tubulin by CC are evident from changes in spectral properties (Andreu and Timashefe, 1981), from immunological reactivities (Morgan and Spooner, 1983) and from polymerization inhibition (Sackett and Varma, 1993).

Calculated spectral parameters S and τ_c were observed to vary with temperature, as expected. These variations are shown in Figs. 3 and 4 for untreated and CC-treated erythrocyte cells. The magnitude of changes in the measured S and τ_c values for untreated and microtubule depolymerizing drug treated (CC) cells were of the same order as those found in the literature for erythrocyte membranes (Minetti and Di Stasi, 1987; Forte *et al.*, 1985; Xiaojie *et al.*, 1989). Since 5-DSA 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 rod-like molecules. However, without obtaining the rotational viscosity, the activation energy of rotational viscosity, was easily obtained from Andrade plots, which showed the existence of phase transition of hydrocarbon chain regions. From Figs. 3 and 4 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 (Minetti and Di Stasi, 1987; Xiaojie *et al.*, 1989; Thomson and Huang, 1980).

Erythrocyte membranes have been reported to undergo more than one thermotropic phase transition using ESR and spin labeled fatty acid labels (Minetti and Di Stasi, 1987; Forte *et al.*, 1985; Wood *et al.*, 1987). Low temperature phase transition was not observed in the present work due to the fact that in the temperature range of 0–20 °C proper determinations of experimentally measured quantities ($T'_{||}$, T'_{\perp} , $h_{(1)}$, $h_{(0)}$, $h_{(-1)}$, $\Delta H(0)$) which were used in Eqns. (1) and (2) to calculate S and τ_c parameters, were not possible. Furthermore, in this temperature range, the validity of the assumptions made in deriving the Eqns. (1) and (2) is questionable.

Our results indicate that: 1. spin labeling is a powerful technique for studying antitubular, that is tubulus destroying, drugs-erythrocyte membrane interactions and support models which predict that such interaction affect membrane dynamic features and its functions; 2. the use of the 5-DSA 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 from the membrane.

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