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Cherman : D.J. Ward (Australia)
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Report Nrº 4

IWTO TECHNOLOGY & STANDARDS COMMITTEE: BRIEFING PAPER

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TITLE: "Thermotropic behaviour of internal wool lipids"

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SUMMARY OF FINDINGS:

Internal wool lipids (IWL) have been used to form stable liposomes, which can be considered as a good model of the wool lipid membrane. Thermotropic physicochemical techniques such as Differential Scanning Calorimetry (DSC) and Electron Paramagnetic Resonance (EPR) have been applied to these liposomes formed with internal wool lipids (IWL) and liposomes formed with Phosphatidylcholine (PC). Differences in fluidity obtained from EPR results permits to deduce a high rigidity of the polar extemai section of the vesicle opposite to the high fluidity of the hydrophobic internal section. The coincidence of the thermotropic changes obtained with the EPR and DSC techniques allows to deduce a partial reversible phase transition temperature on the range of 30 to 40°C, temperatures lower than phase transition temperatures of lipids from other keratinized tissues but higher than PC liposomes.

COMMERCIAL IMPLICATIONS – CURRENT& FUTURE:

It is generally accepted that cell membrane lipids of wool govern the permeability of the fibre, being fundamental in its dyeing and diffusion properties. Wool fibre is histologically constituted of cuticle and cortical cells held together by the cell membrane complex which forms the only continuos phase in keratin fibre. The influence of this minor component, especially the lipids, on the physical, chemical and mechanical properties of wool fibres is well known.

The bilayer-forming capability of internal wool lipids and some physicochemical properties have been studied in an attempt to enhance our understanding of the lipid structure present. Internal wool lipids were shown to form stable liposomes, however not much work has been done studying thermotropic phase behaviour of these components that can be critical in the diffusion properties of the wool fibres.

Thermotropic behaviour of internal wool lipids will adduce new information about the fluidity and structure of the lipid bilayers of the liposomes made with intemai wool lipids. Advances in the knowledge of internal wool lipid arrangement will help to understand the behaviour of this minor fraction which is so important on the different states of the wool processing.

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THERMOTROPIC BEHAVIOUR OF INTERNAL WOOL LIPIDS

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SUMMARY

Thermotropic physicochemical techniques such as Differential Scanning Calorimetry (DSC) and Electron Paramagnetic Resonance (EPR) have been applied to liposomes formed with internal wool lipids (IWL). These vesicles can be considered as a model of wool membranes. Differences in fluidity obtained from EPR results permits to deduce a high rigidity of the polar external section of the vesicle opposite to the high fluidity of the hydrophobic internal section. The coincidence of the thermotropic changes obtained with the EPR and DSC techniques allows deducing a partial reversible phase transition temperature on the range of 30 to 40°C.

1. INTRODUCTION

It is generally accepted that cell membrane lipids of wool govern the permeability of the fibre, being fundamental in its dyeing and diffusion properties. Wool fibre is histologically constituted of cuticle and cortical cells held together by the cell membrane complex which forms the only continuous phase in keratin fibre. The influence of this minor component, especially the lipids, on the physical, chemical and mechanical properties of wool fibres is well known (1).

The internal wool lipid material (IWL), which accounts for only about 1.5% of total fibre weight, consist of three major lipid classes; sterols, fatty acids and polar lipids. The sterols consists predominantly of cholesterol, desmosterol and sterol esters. The major fatty acids are stearic, palmitic, oleic and 18-methyleicosanoic. The polar lipids consist predominantly of ceramides, cholesterol sulphate and glycosphingolipids, only traces of phospholipids have also been detected (2-4). This lipid composition do much resemble to those found in membranes from other keratinized tissues such as stratum corneum

of skin or human hair.

The thermotropic phase behaviour and structural parameters of lipids constituting the intercellular cement of human stratum corneum have been a subject of investigation over the last decade. Such studies based on differential scanning calorimetry (DSC), infrared spectroscopy, or X-ray diffraction analysis can indeed provide important insight into the function of these lipids and their role in the interactions of the stratum corneum with xenobiotic chemicals (5-8).

Liposomes made up of stratum corneum lipids have been widely used as a model of skin lipid bilayer, whilst considering possible interaction mechanisms between liposomes and several compounds especially surfactants (9-10) and retarders or enhancers (11-13). Changes in the lipid order have been related to the modification of their fluidity. This could be associated with the role of this lipid structure in skin permeation.

In the case of wool, despite the advances in characterizing the lipid composition of the cell membrane complex of wool, little progress has been made in furthering our understanding about the structure, arrangement and thermotropic behaviour of these components. The cell membrane complex has different CMC regions according to their dyeability. There are two resistant membranes, two unstained layers called the β-layers, and a dark stained central layer, the δ-layer. The β-layers, generally believed to be made up of lipids, are assumed to form a bilayer structure.

In fact, the bilayer-forming capability of internal wool lipids and some physicochemical properties have been studied in an attempt to enhance our understanding of the lipid structure present. Internal wool lipids were shown to form stable liposomes, however not much work has been done studying thermotropic phase behaviour of these components that can be critical in the diffusion properties of the wool fibres (14,15).

This work has been focused on the formation of liposomes as a model of a wool lipid membrane and their thermotropic physicochemical properties. We have used Quasielastic Light Scattering (QELS) to determine size distribution, Differential Scanning Calorimetry (DSC) to obtain thermal transition temperature and spin label Electron Paramagnetic Resonance (EPR) techniques to monitor the molecular dynamics of lipids.

Many of these physical techniques have been extensively used in characterising phospholipid vesicle systems(16,17) and in the last decade they have also been applied to stratum corneum lipid systems (18-20); however they have not been applied to wool lipid systems.

Specially, EPR permits to detect changes in the spin tropic movement of an unpaired electron. Biomolecules like IWL that do not contain unpaired electrons, can be studied by EPR when they are surrounded or chemically bonded to stable free radical. This radical, or spin label, produces a sharp and simple EPR spectrum that gives detailed information concerning the molecular environment of the label (21,22). The doxyl stearic acid labels (DSA), could have great efficiency for the IWL study when they are structured in liposomical form. These compounds are formed by a radical group (doxyl)

and a hidrocarbonated chain (stearic acid) which acts of radical support (Fig.1) and functionality of the alkyl chain is very similar to lipids of the IWL bilayer, therefore a good interaction could be envisaged.

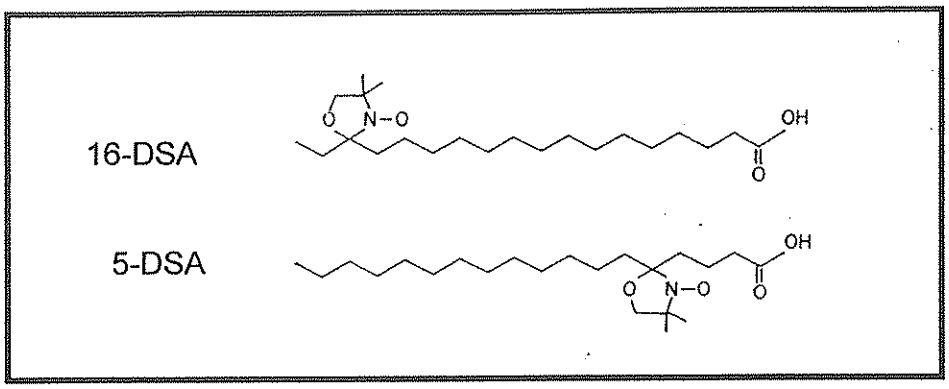

Figure 1. Molecular formula of the spin probes, 5 and 16 doxyl stearic acids

Furthermore, the radical position in the alkyl chain will determine motional profiles of the different bilayer levels where the nitroxide label is integrated. The doxyl nitroxides used in present work, 5-DSA, with the radical near the polar headgroup and 16-DSA with the radical at the end of the hydrophobic chain were selected to determine the fluidity of the different regions of the bilayer since the spin probes are oriented and linked like the lipids in the bilayer (23,24).

Application of this EPR technique with the different spin labels varying the temperatures will adduce new information about the fluidity and structure of the lipid bilayers of the liposomes made with internal wool lipids. Advances in the knowledge of internal wool lipid arrangement will help to understand the behaviour of this minor fraction so important on the wool permeation.

2. EXPERIMENTAL

2.1. Materials

Raw industrially scoured Spanish Merino wool supplied by Corcoy S.A. (Terrassa, Spain) was used to obtain the internal wool lipids. Prior to the extraction, wool was equilibrated in a conditioned room (20°C, 60% relative humidity).

The chemicals were analytical grade and the standards used were supplied by Sigma Co (St. Louis, M.O.) in the case of ceramides type III and cholesterol sulphate and by Fluka (Buchs, Switzerland) in the case of cholesteryl-palmitate, palmitic acid and cholesterol. 5-doxyl stearic acid (5-DSA) and 16-doxyl stearic acid (16-DSA) were also obtained from Sigma Co (St. Louis, M.O.). Lipoid S-100, whose main component is soy bean phosphatidylcholine (>95%) was obtained from Lipoid (Germany). All these chemicals were stored in chloroform/methanol 2:1 under frozen temperatures until use. Tris(hydroxymetihyl)-aminomethane (TRIS buffer) supplied by Merck (Darmstadt, Germany) was prepared as 5 mM TRIS buffer adjusted to pH 7.40 with HCl, containing 100 mM of NaCl.

2.2. Methods

Preparation and characterisation of liposomes

The internal lipids were soxhlet extracted from cleaned wool (4gr) with chloroform/methanol azeotrope (250mL, 79:21 v/v) for 5 hours. The lipid extracts were concentrated down to 10 ml under a stream of dry nitrogen and stored in 2/1 chloroform/methanol at 6°C. Aliquots were dried and weighed and the lipid percentage extractions were quantified (3,25). Quantitative analysis was performed using Thin-layer chromatography (TLC) coupled to an automated ionisation detection (FID) system (Iatroscan MK-5, Iatron Lab. Inc. Tokyo, Japan) with a Sample Spotter SES 3202/IS-01 (SES GmbH, Nieder-Olm, Germany) using an optimised analytical procedure (15).

Samples of 1 mL of IWL solution (10mg/mL chloroform/methanol 2:1) were taken to dryness in culture tubes with a stream of nitrogen. 1ml of buffer containing 100 mM NaCl, 5 mM TRIS was added, to provide a final concentration of approximately 10 mg lipid per ml. Suspensions were then sonicated in a sonicator Labsonic 1510 (B. Braum) at 100 Watt with a thermostated bath Ultraterm 6000383 (Selecta) at a temperature of 65^{0}C for about 15 min until the suspensions became homogenoeus. The preparations were then annealed at the same temperature for 30 min and incubated at 37°C under nitrogen atmosphere.

Phosphatidylcholine (PC) liposomes were also prepared with Lipoid S-100 using the same methodology already described to achieve also a final lipid concentration of 10 mg per mL in TRIS.

Mean vesicle size distribution and polydispersity indexes of the liposomes were determined using a Photon correlator spectrometer (Malvern Autosizer 4700c PS/MV) by particle number measurement at 37°C with a lecture angle of 90°.

Differential Scanning Calorimetry

The thermal behaviour of lipid samples was studied with a DSC apparatus MicroCal MC 2 microcalorimeter (MicroCal, Inc., Northampton, MA). Data were processed with Origin software. The liposome suspension of IWL was introduced into the chamber cell at a final IWL concentration approximately of 5 mg/mL in TRIS buffer. The same medium was placed in the reference cell .The sample was equilibrated to 10°C and then heated from 15 to 100°C at a rate of 40°C/h, and then cooled back to 10°C at the same rate.

A second and third cooling and heating processes were performed and the heating thermograms were recorded from 15 to 100°C, to determine whether or not the observed thermal transitions were reversible. Thermograms of TRIS buffer were taken as instrumental baseline and subtracted from sample scans.

Electron Paramagnetic Resonance

Electron Paramagnetic resonance (EPR) techniques are used to monitor the molecular dynamics of lipids. Like the RMN, it is a spectroscopic technique based on the

interaction between the electron magnetic moment and an externally magnetic field that permits to study the behaviour of molecules with unpaired electrons (paramagnetic molecules). Two possible rotation senses of the spin magnetic moment, in favour or against the field sense, will produce two stationary states of different energy, separated by an energy interval (Zeemann's energy) that it is function to the value of the foreign magnetic field applied (26,27).

The EPR apparatus irradiates with an electromagnetic radiation of determined frequency a sample labeled with a paramagnetic radical. At the same time, a powerful magnetron submits the sample to a sweep of magnetic field intensity. When the value from the Zeemann energetic jump produced by the magnetic field coincides with the energy of the radiation irradiated by the source, a change in the spin direction takes place which is registered in the spectrum as a peak (or a double peak, when the first derivative is performed).

EPR spectra of spin labels included in the membrane are sensitive to the mobility of the adduct between bilayer and spin labels, the polarity of the environment surrounding the spin labels and the orientation of the spin labels. Therefore, the orientation of labels in bilayers must reflect the local molecular structure and should serve as delicate indicators of conformational changes in bilayers (28). The EPR spectra study permits to calculate the order parameter (S), related with the bilayer-labeled fluidity. In some circumstances, when the motion of label appears to be predominantly isotropic, the well-known order parameter no longer applies (21), and an empirical parameter, the rotational correlation time (τ), can be used to give a measure of motion in the region probed by the spin label (29,30). A first-derivative of an electron spin resonance spectrum and the equation to obtain the rotational correlation time parameter can be visualised in Figure 2. The W_0 parameter or line width in G and the heights of the mid- and high-field lines can also be seen in this Figure.

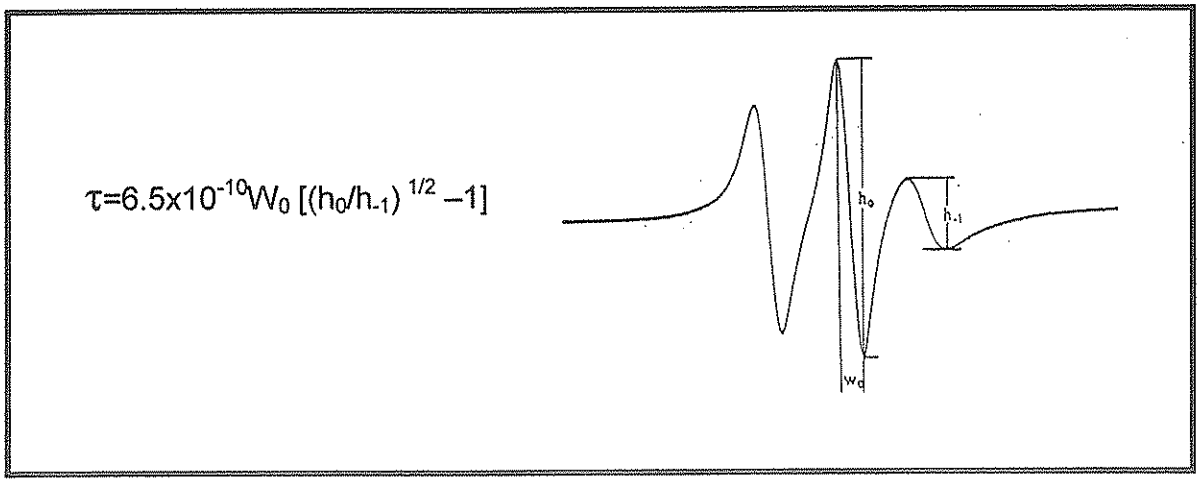

Figure 2. Electron paramagnetic resonance spectrum of 5-DSA in Tris buffer solution, and the mathematical formula to obtain τ .

In the present work, EPR measurements were performed using a Varian E-109 spectrometer (Varian Associates, Palo Alto, CA) working in the X-band and equipped with a Varian E-257 temperature control unit. The hyperfine splitting of labeled liposome samples were determined with conditions of 100G scan width, 3360G of field intensity, 8 minutes scan time and 5mW microwave power.

Liposome samples were labeled with 5- and 16-doxylstearic acid (5-DSA and 16-DSA) adding the appropriate amount of spin labels to the lipids before taken to dryness. Liposomes were then performed as previously described to achieve a final label concentration of $3x10^{-4}$M. Control samples ($2x10^{-4}$ M, 5-DSA and 16-DSA in TRIS buffer) were prepared in tandem with the spin labeled liposome samples.

The EPR measurements were carried out with a standard quartz EPR tube of 21 cm in length and 1.5 mm of inner diameter. The spin-labeled liposome sample was placed in the EPR cavity for 2 min before the data acquisition to assure thermal equilibration and temperature was measured before and after the spectrum recording to assure thermal constancy. After each EPR measurement, the sample was removed from the cavity and placed in the water bath at the same temperature until the cavity reached a new temperature equilibrium. Data were collected in the temperature range interval from -25 to 75°C. Line width in G, W_0 and heights of the mid- and high-field lines, h_0 and h_{-1} respectively were obtained from the first-derivative of each absorption spectrum and the rotational correlation time (τ) was calculated applying the equation from Figure 2.

3. RESULTS AND DISCUSSION

Internal wool lipids were obtained by extraction in soxhlet with chloroform/methanol azeotrope obtaining an amount of 1.2% on wool weight. The application of the TLC/FID technique to these fractions enabled us to quantitatively determine their composition. The percentages obtained of the major lipid classes are 10% of sterol esters, 24% of free fatty acids, 12 % of cholesterol, 46% of ceramides and 8% of cholesteryl sulphate.The lipids from other keratinized tissues such as stratum corneum also consisted of the same kind of compounds, the main difference being the higher amount of cholesterol (25%) (31,32).

Liposomes were prepared with this internal wool lipid and the particle size distribution of the IWL liposome suspensions was determined at one and twenty-four hours after vesicle preparation. A particle size average mean distribution of about 270 nm and a polydispersity index around 0.2 was found at the two different times. The stability of this suspension was shown to be longer than a week.

EPR thermobehaviour of the liposome suspensions was determined as described in the experimental part. The EPR spectra study together with the mathematical formulation developed by Keith & al.,(29) $\tau = 6.5x10^{-10}W_0 [(h_0/h_{-1})^{1/2} -1)$, permits to calculate the τ parameter, rotational correlation time. This parameter describes the freedom degree of the radical spin movement. Generally, a direct relation is established between the spin movement and the environment viscosity in which the label is

surrounded, therefore it is possible to relate it with the fluidity of the labeled system.

In the present work two different spin-labels are applied, 5-DSA and 16-DSA to study the liposome fluidity close to the polar surface region and near the central region of the bilayer respectively. EPR spectra of control samples in TRIS buffer, of IWL liposomes and of PC liposomes were obtained in duplicate at different temperatures and the rotational correlation times calculated are listed in Table 1.

Table 1. Rotational correlation time parameters calculated from the EPR spectra obtained at different temperatures for the 5-DSA and 16-DSA labeled samples (Control inTRIS buffer, IWL liposomes and PC liposomes)

T(°C)	Rotational Correlation Time					
	5-DSA			16-DSA		
	TRIS	IWL	PC	TRIS	IWL	PC
-25	--	11.21	12.20	--	--	4.31
-10	*	11.75	9.49	*	--	2.15
0	0.65	11.68	5.40	0.15	--	1.70
10	*	11.70	3.72	*	--	1.46
25	0.23	6.86	3.43	0.08	3.90	0.85
35	*	4.85	2.83	*	2.38	0.64
45	0.08	3.40	2.18	0.04	1.46	0.46
55	*	2.75	1.96	*	1.11	0.35
65	0.05	2.19	1.67	0.04	0.67	0.31
75	*	1.56	1.38	*	0.50	0.28

* Not performed

-- Difficult to calculate due to low signal-to-noise relation

In some cases at low temperatures the τ parameter could not be calculated since the freezing of the sample sometimes prevents to obtain spectrums with an acceptable signal-to-noise relation. Since the rotational correlation time is directly related with the structural order, a decrease of this parameter τ (increase of the fluidity) is obtained for all samples with an increase of temperature. Furthermore, in all cases samples labeled with 5-DSA have higher correlation time than samples labeled with 16-DSA. This must be due to the lower fluidity of the environment of the polar groups evaluated by the 5-DSA spin label. In Fig. 3 the variation of the correlation time parameter, τ with the temperature of control and IWL liposome labeled samples is represented.

EPR spectra of TRIS buffer samples show a much lower signal than the IWL spin-labeled samples. This was expected due to the high fluidity of the labels on the liquid system at temperatures higher to 0°C. Furthermore, this fact would suggest that the amount of free spin probe in samples do not much affect to the calculation of the τ parameter values (in all cases τ values of liposome samples are more than ten times higher than the τ values of buffer samples).

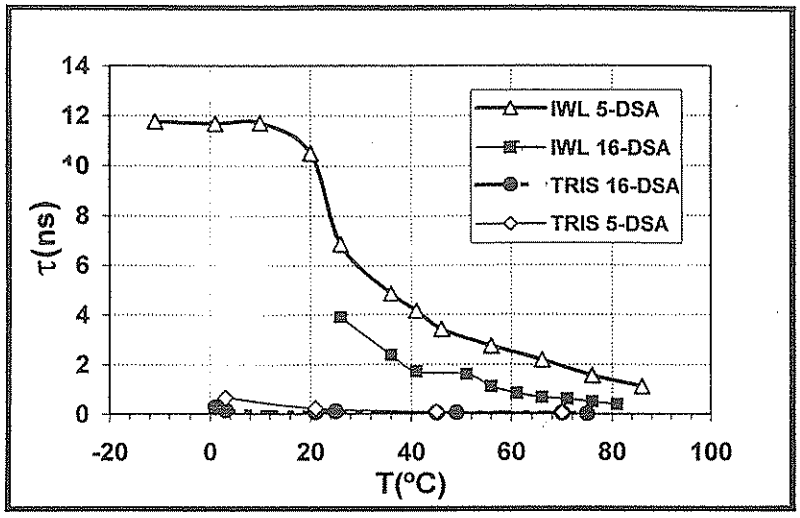

Figure 3. Rotational correlation time τ, of 5-DSA and 16-DSA in control TRIS buffer samples and IWL liposomes, versus temperature.

The curve of 5-DSA labeled liposomes, have much higher values of τ parameter than that observed in 16-DSA labeled liposomes at the same temperatures. In fact, it seems to be in agreement with earlier works in which EPR results indicated more restricted motion near the polar headgroup region than near the centre of alkyl chain region. This situation may be explained if we bear in mind the different chemical configuration of the involved groups in the bilayer conformation.

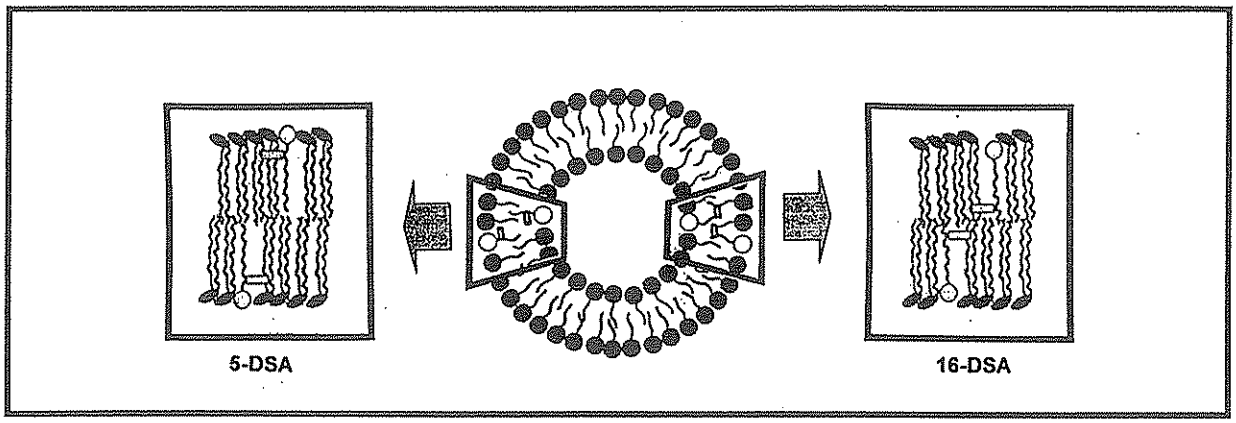

Figure 4. Sectional view of a liposome with 5-DSA and 16-DSA spin labels integrated in the bilayer.

A sectional view of a liposomal bilayer (Fig. 4) shows the polar extremes of fatty acids grouped on the surface of the bilayer in which the nitroxide group of 5-DSA label is placed. These polar groups make interactions between them as well as with polar molecules of involving medium. These hydrophilic interactions by hydrogen bonds or ionic bonds conform a stable external layer of great cohesion.

In a more internal level we find the alkyl chains of the fatty acids, arranged in parallel, establishing between them apolar interactions of Van der Waals. However, the centre of the bilayer, in which the nitroxide group of 16-DSA is placed, is characterised to be the region with smaller cohesion of all the liposomal structure, at the end of the monolayers. Cytological studies have demonstrated that the monolayers of the

citoplasmatic membrane in animal cells, of very similar structure that the liposomes of study can rotate in opposite sense at physiological temperature. On the other hand, experiments with electron microscopy have shown the presence of arranged fatty acids lengthwise of this zone of monolayers union, unstable arrangement for the liposome since they are in close contact polar groups in dominance apolar zones.

The thermotropic behaviour of IWL liposome labeled samples can be also visualised in Figure 3. There is an important jump in τ values in the temperature range from 20 to 40°C in both spin-labeled samples. This rapid change in the fluidity of the system in a determined temperature range is explained as a modification of the structure of the liposome produced in a phase transition temperature in which different physicochemical properties change.

Differential Scanning Calorimetry (DSC) is the most appropriate technique with high capacity to quantify direct and precisely, small enthalpic variations, associated with structural changes, by the action of the temperature. Therefore, thermograms were carried out to confirm if the inflection point of EPR curves, might be due to a change in the structure of the liposome. IWL liposome samples were calorimetrically scanned three consecutive times and the enthalpic changes detected from 15 to 100°C can be observed in Figure 5.

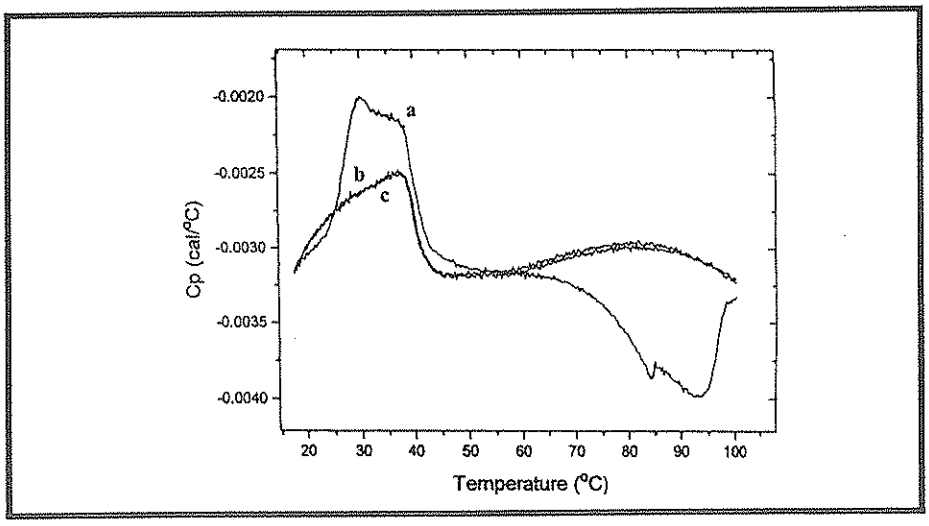

Figure 5. Differential Scanning Calorimetry (DSC) of three consecutive thermograms a, b and c of IWL liposomes.

In the first warming process (curve a) there is a broad endothermic peak well delimited from 20 to 40°C and a second exothermic peak, that begins at 60°C and it is maintained from 83 to 96°C. In a second heating process of the sample (curve b) the endothermic peak appears at the same temperature range, from 20 to 40°C, but with smaller intensity and the exothermic peak has disappeared. In the third heating thermogram, the endothermic transition was essentially unchanged and reproducible, indicating that this enthalpic transition is partially reversible.

The behaviour of these thermal transitions show the existence of three states of the IWL liposomes in a temperature range from 15 to 100°C, with a main transition centred

at about 30-40°C, and a second transition at about 80-90°C. However, after reheating the thermal transitions obtained indicate the irreversible character of the process. It could be suggested that an interaction of low cooperativity may take place between the lipid fraction responsible of part of the main transition phase and the second one. These new low cooperativity interactions between lipids might be responsible of the irreversibility of part of the first thermal transition and the second one.

These transitions have been attributed to alkyl chain melting (33). Broad transitions are characteristic for lipid mixtures containing cholesterol like in the IWL liposomes (34-36). It is possible to suggest a correspondence between the change on the fluidity and on the enthalpy of the system, in a temperature range below 40°C, being able to conclude that it is a temperature range where the IWL structured in bilayer form undergo a thermotropic change. This value is slightly lower than the value obtained for pig stratum corneum lipids (65°C (32)) or human horny layer lipids (75°C (37)). This difference could be due to the different lipid composition, taking into account the lower amount of cholesterol present in the IWL liposomes comparatively with SC.

However, this temperature range it is much higher than the phase transition temperature of lipid mixtures usually used to form liposomes, such as phosphatidylcholine, below 0°C. The properties and characteristics about the phosphatidylcholine and their capacity to form stables liposomes with a transition phase temperature (below 0°C) are very known. Therefore, EPR was also performed with phosphatidylcholine liposomes labeled with the same spin labels 5-DSA and 16-DSA to determine the fluidity changes of these structures and their relation with phase transition temperatures obtained by DSC.

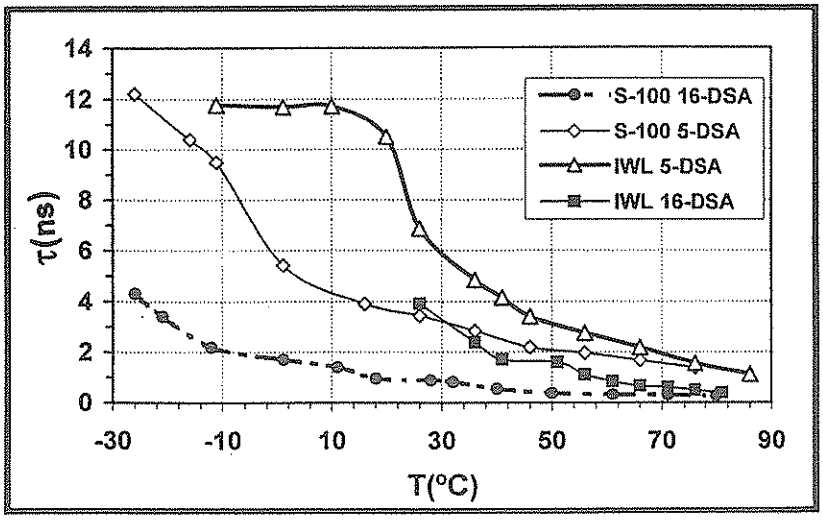

Figure 6. Rotational correlation time τ, of 5-DSA and 16-DSA in IWL liposomes and PC liposomes, versus temperature.

In Figure 6, the variation of the τ parameter for IWL liposomes and PC liposomes labeled is represented as a function of temperature. It can be seen that a change of fluidity appears at temperatures below 0°C, in 16-DSA as well as in 5-DSA spin labeled PC liposomes. The PC labeled liposomes have lower values than the IWL liposomes at

the same temperatures. The main reason that would explain this higher fluidity in PC labeled liposomes would be the lack of rigid molecules as cholesterol and some other big chemical compounds, that would difficult by steric hindrance the possible tropic movement of the label. Furthermore, the several insaturations in the phosphatidylcholine chain produce disorders in the lipid structure allowing free movements of the alkyl chains

CONCLUSIONS

Internal wool lipids (IWL) have been used to form stable liposomes, which can be considered as a good model of the wool lipid bilayer membrane. Thermotropic physicochemical techniques such as Differential Scanning Calorimetry (DSC) and Electron Paramagnetic Resonance (EPR) have been applied to these liposomes formed with internal wool lipids (IWL) and liposomes formed with phosphatidylcholine (PC). Differences in fluidity obtained from EPR results permits to deduce a high rigidity of the polar external section of the vesicle opposite to the high fluidity of the hydrophobic internal section. The coincidence of the thermotropic changes detected for the IWL liposomes with the EPR and DSC techniques allows to deduce a partial reversible phase transition temperature on the range of 30 to 40°C lower than phase transition temperatures of lipids from other keratinized tissues but higher than PC liposomes. This specific behaviour may be explained by the complex chemical nature of the internal wool lipid material used for preparation of liposomes.

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