THE 9TH INTERNATIONAL WOOL TEXTILE RESEARCH CONFERENCE

28TH JUNE - 5TH JULY, 1995
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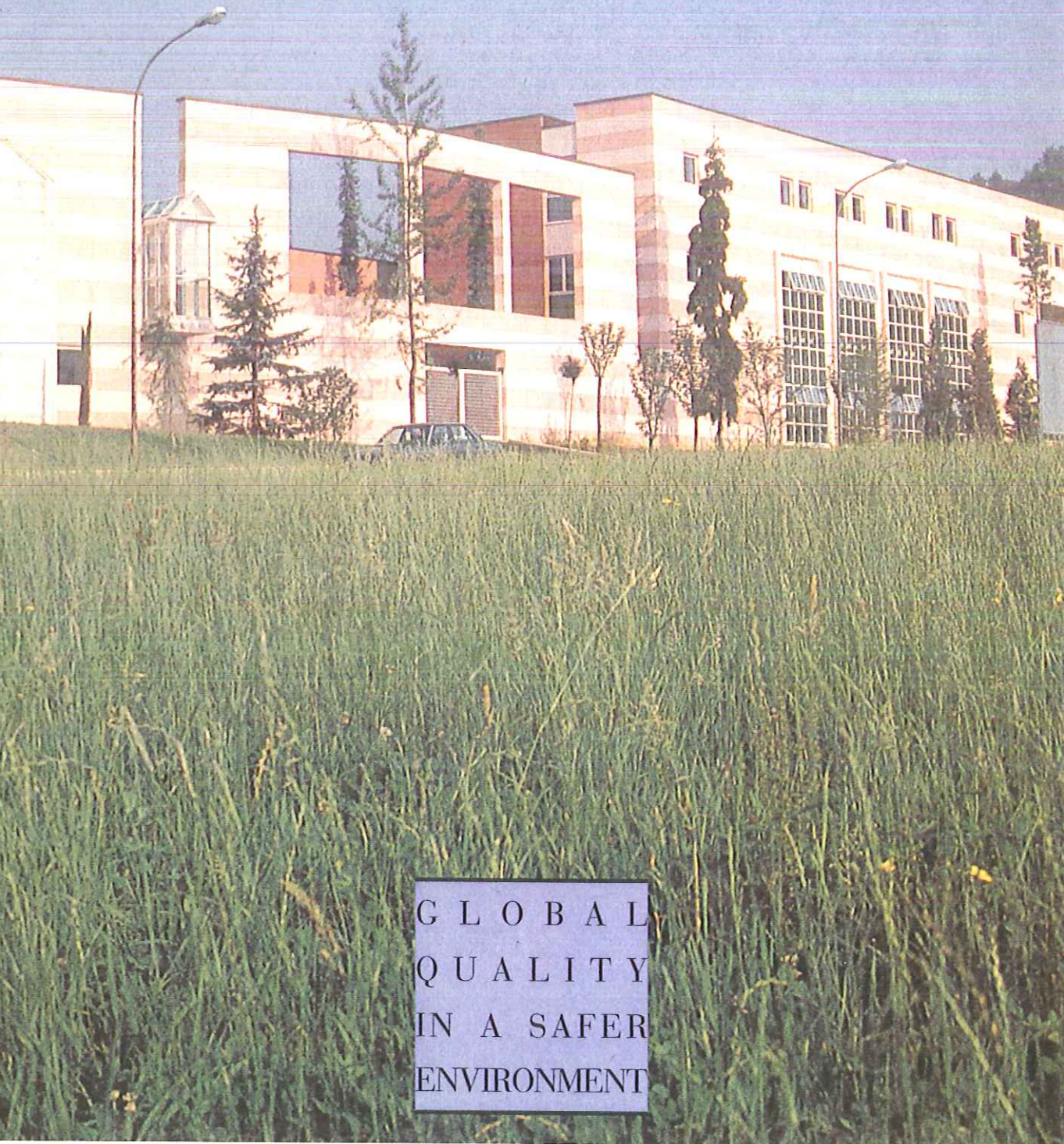

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PROCEEDINGS OF THE 9TH INTERNATIONAL WOOL TEXTILE RESEARCH CONFERENCE

VOLUME II

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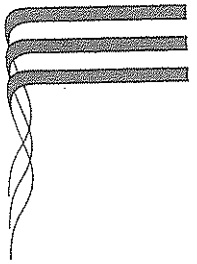

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VOLUME II

Wool structure and properties
Protein chemistry
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WOOL STRUCTURE AND PROPERTIES

LIPOSOME FORMATION WITH INTERNAL WOOL LIPIDS

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SYNOPSIS

The bilayer-forming capability of internal wool lipids and their physico-chemical properties were studied to enhance our understanding of the lipid structure present in wool and other keratinized tissues. Internal wool lipids were extracted and quantified and the mixture obtained was shown to form stable liposomes with a phase transition temperature of 60°C. The spontaneous permeability of these vesicles was found to be very small, similar to vesicles formed with lipids from other keratinized tissues. The TEM micrographs showed big vesicle structures of approximately 300nm in size, which seem to be made up of smaller structures of approximately 20nm in size. This particular structure could account for the high diameters and small internal volumes found by other methodologies.

INTRODUCTION

The Cell Membrane Complex (CMC) of wool is known to play an important role in the adhesion between cell of both the cuticle and the cortex in the keratinized fibre in the transport of dyestuffs and processing chemicals into the fibre and in the determination of the surface properties of the fibre [1].
The CMC is made up of lipids and proteins, which are also the primary constituents of plasma membranes of living cells. Merino wool fibres contain about 1% by weight of lipids. The major lipids present in the CMC of wool (sterols, fatty acids and ceramides) [2, 3], do not resemble those normally found in viable cells, such as phospholipids, but those found in the membranes of the stratum corneum of skin.
Despite recent advances in the characterization of the lipid components of the CMC [1-4], little progress has been made in increasing our understanding of the structure and arrangement of these components within the CMC. The β-layers of the CMC are assumed to be due to lipid bilayers, derived from the plasma membranes of living cells. The types of lipids, low levels of phospholipids found in the CMC and observations that phospholipids are lost in keratinisation of skin [5] suggest, however, that the lipid bilayers could be fairly different from those in plasma membranes.
The lipids of the stratum corneum are capable of forming liposomes [6] even when phospholipids are not present. Thus, the β-layers in the CMC might contain a lipid bilayer in a manner simmilar to the multiple intercellular lipid lamallae formed in the stratum corneum of mammals [7]. In fact, liposomes have been reported to be formed from hair and wool lipids [8].
Previous work carried out in our laboratories on the formation of stratum corneum lipid liposomes involved the study of the influence of the lipid composition on the bilayer formation and their physico-chemical properties [9]. The cholesteryl sulfate component was found to play an important role, especially in the permeability modification of these structures.
Hence, we investigate the bilayer-forming capability of internal wool lipids, and their physico-chemical properties, especially their permeability.

Comparison of these results with the ones obtained with the bilayers formed with stratum corneum lipids and with phospholipids could improve our understanding of the lipid structure present into the keratinized tissues.

EXPERIMENTAL

Materials

Raw industrially scoured Spanish Merino wool supplied by Corcoy S.A. (Tarrasa, 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 sulfate and by Fluka (Buchs, Switzerland) in the case of Cholesteryl-palmitate, palmitic acid and cholesterol.
The nonionic surfactant Triton X-100, octylphenol polyethoxilated with 10 units of ethylene oxide and active matter of 100% was purchased from Rohm and Haas (Lyon, France). Tris-(hydroxymethyl)-aminomethane (TRIS) was supplied by Merck. Fluorescent 5(6)-carboxyfluorescein (CF), (Eastman Kodak, Rochester, NY) was purified according a the column chromatographic method (10). The buffer used was 5.0 mM TRIS with 100mM NaCl (reagent grade) adjusted to pH 7.50 and supplemented with 10 mM CF when studying bilayer permeability of liposomes. Water was also purified by a Milli-Ro system (Millipore, Madrid, Spain).

Methods

Isolation and analysis of internal wool lipids

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, 11].
Qualitative lipid analysis was performed as described in an earlier work [3]. The quantitative analysis was performed using Thin-layer chromatography (TLC) coupled to an automated ionization detection (FID) system (Iatroscan MK-5, Iatron Lab. Inc. Tokyo, Japan) [12, 13].
The lipid fractions were directly spotted (1µl) with a Sample Spotter SES 3202/IS-01 (SES GmbH, Nieder-Olm, Germany) on Silica Gel SIII Chromarods and developed with the first solvent system i) n-hexane/ethyl ether/formic acid (50:20:0.3, v/v/v) to separate the non-polar lipids. After partial scan of 80% to quantify and eliminate the apolar lipids, the redevelopment of Chromarods with ii) chloroform/methanol/ammonia (58:10:2.5, v/v/v) twice for 7 cm, leads to the separation and quantification of the polar lipids [3, 14]. The same procedure was applied to the standards cholesterol palmitate, palmitic acid, cholesterol, ceramide III, and cholesterol sulfate to determine their calibration curves for the quantification of each compound.

Preparation of liposomes

Liposomes were formed by internal wool lipids (IWL) extracted and analyzed as previously described. 0.5 mL of IWL solution (≅10mg/mL chloroform/methanol 2:1) were taken to dryness in culture tubes with a stream of nitrogen. Four ml of buffer containing 100 mM NaCl, 5 mM TRIS and supplemented with 10 mM CF dye to provide the final concentration of approximately 1 mg lipid per ml at pH 7.50 were added. Suspensions were then sonicated in a sonicator Labsonic 1510 (B. Braum) at 100 Watt with a thermostated bath Ultraterm 6000383 (Selecta) at the temperature of 65°C (5°C higher than that corresponding to phase transition temperature of IWL, see Results) for about 15 min until the suspensions became clear. The preparations were then annealed at the same temperature for 30 min and incubated at 37°C under nitrogen atmosphere.

Characterization of Liposomes

Phase transition temperature
Wool internal lipids were left to hydrate for 10 min at 80°C in 1mL deuterated water. Suspensions were then sonicated in a bath sonicator at 80°C for 10 min. Proton magnetic resonance spectra were obtained at a temperature ranging from 25-90°C using a Varian Unity of 300 MHz to determine the lipid phase transition temperatures of the lipid dispersions forming liposomes. The line widths of the CH_2 band at 1.3 ppm were measured after 1024 scan accumulation.

Determination of permeability alterations, encapsulation efficiency of bilayers and internal volume
The complete liposome suspensions were chromatographed through Sephadex G-50 medium resin (Pharmacia Uppsala, Sweden) with TRIS buffer and the liposome fraction freed of unencapsulated material was kept to 100 mL with also TRIS buffer to measure the liposome permeability spectrofluorimetrically by means of the CF release.
The spontaneous permeability alterations of the IWL liposomes versus time was determined by monitoring the release of the CF from these structures [15]. Fluorescence measurements were made at different times with a Shimadzu RF-540 spectrofluorophotometer equipped with a thermoregulated cell compartment (λ_{ex}495 nm, λ_{em}515.4 nm).
The proportion of the CF released was calculated by means of the following equation (10):

$$\%\text{CF release} = \frac{I_t - I_0}{I_\infty - I_0} \cdot 100$$

where I_0 is the initial fluorescence intensity of the CF-loaded liposome suspension at 515.4 nm and I_∞ is the fluorescence intensity at 515.4 nm after destroying the liposomes by the addition of Triton X-100 (60µl of 10% (v/v) solution). I_t corresponds to the fluorescence intensity at different time intervals.
The encapsulation efficiency defined as the fraction of the aqueous compartment sequestered by bilayers and expressed as % was calculated from the I_∞ value.

The internal volume of liposomes defined as the volume enclosed by a given amount of extracted material and expressed as ml/mg was calculated from the ratio between the encapsulation efficiency value and the lipid amount in the liposome suspension.

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

Electron microscopy
Freeze Fracture: Liposome suspensions were placed on thin copper specimen carrier plates and frozen in liquid propane at -190°C. Freeze-fracturing was carried out in a Balzers 301 apparatus (Balzers AG, Balzers, Lichtenstein), and the specimen was shadowed with platinum and coated with carbon. The replicas were then coated with a support film of Parlodion applied in amyl acetate and air-dried before the copper carriers were dissolved by floating in an acid mixture (orthophosphoric:sulfuric: glacial acetic, 1:1:1). The replicas were then washed in distilled water, cleaned in Clorox bleach for 2-3 h, and rinsed several times in distilled water before being picked up on Formvar-coated grids. The Parlodion support film was dissolved by standing in methanol for 30 min. The cleaned replicas were examined in a Hitachi H-600 AB transmission electron microscope operating at 75 kV.

Negative Staining: Carbon-coated copper/palladium grids G-400 mesh, 0.5 Taab with 0.5% E 950 collodium films in n-amylacetate were employed. A drop of the vesicular solution was sucked off the grid and after 1 minute with filter paper down to a thin film. Negative staining with a drop of a 1% solution of uranyl acetate was performed. After 1 minute this drop was again removed with filter paper and the resulting stained film was dried in a dust free place. Samples were also examined in a Hitachi H-600 AB transmission electron microscope operating at 75 kV.

RESULTS AND DISCUSSION

Lipid analyses

Internal wool lipids were obtained by extraction in soxhlet with chloroform/methanol azeotrope. The yield of lipids obtained using this method was 1.2% on wool weight. Internal wool lipids are believed to account for similar weight percentages, (0.8 [3], 1.2% [16] and 1.5% [1]).
The application of the TLC/FID technique to these fractions permitted us to quantitatively determine their composition. The method consists in a multiple development of Chromarods with solvent systems described in the experimental part using a partial scan to solve the non-polar lipids and after redevelopment a total scan for the polar lipids. The same procedure was applied to the standard compounds obtaining the following response factors: SE $0.40.10^{-3}$, FFA $0.35.10^{-3}$, CHOL $0.24.10^{-3}$, CA $0.30.10^{-3}$ and CHOL-S $0.92.10^{-3}$.
The percentages on total lipid extract were obtained from the amount of each compound after multiplying each area by the corresponding response factor.

The mean value of five lipid analyses of the lipid wool extract gave the lipid percentages of the major lipid classes expressed in Table I.

TABLE I - Percentages of internal wool lipids determined by TLC/FID.

COMPOSITION OF INTERNAL LIPIDS OF WOOL (% weight)	
STEROL ESTERS (SE)	9.7
FREE FATTY ACIDS (FFA)	23.6
CHOLESTEROL (CHOL)	11.5
CERAMIDES (CA)	46.4
CHOLESTEROL SULPHATE (CHOL-S)	8.8

Similar values were obtained for the composition of internal wool lipids [3, 13] even though higher cholesterol percentages have been obtained by other authors [2, 12].

Liposome formation and characterization

Liposomes were prepared with the internal wool lipid (IWL) composition listed in Table I following the method described in the experimental part. The liposome formation is not surprising since the percentage of free fatty acids 23.6% and cholesteryl sulfate 8.8% fall in the range that are capable of forming vesicles [6, 9].
The lipid mixture chosen as a model of lipids of another keratinized tissue such as stratum corneum [6] has a similar composition in free fatty acids (25%), ceramides (40%) and cholesterol sulfate (10%); nevertheless, this mixture has a higher amount of free cholesterol (25%). The presence of either free fatty acids or cholesteryl sulfate, which are ionized at physiological pH, was considered to be essential for bilayer formation [6]. Furthermore, lipid mixtures with the same compounds but at the different relative concentrations indicated below, were also capable of forming liposomes [9]: palmitic acid (21.25 to 28.75%), cholesterol (21.25 to 28.75%), ceramides (34 to 46%) and cholesteryl sulfate (0.25 to 19.75). All lipid mixtures reported led to bilayer formation even though their physicochemical characteristics were found to be dependent on the lipid composition used.
Minor differences on the permeability of the vesicles are expected to be found owing to the lower amount of cholesterol, which is known to affect the fluidity of the membrane (17).

The phase transition temperature of the IWL liposome suspension was determined as described in the experimental part. In Figure 1 the different line widths of the CH_2 band of the RMN spectra can be observed at four of the temperatures investigated.
The different line widths represented versus the temperature gave an inflexion point at 60°C for the IWL which can be taken as the phase transition temperatures for this lipid mixture.

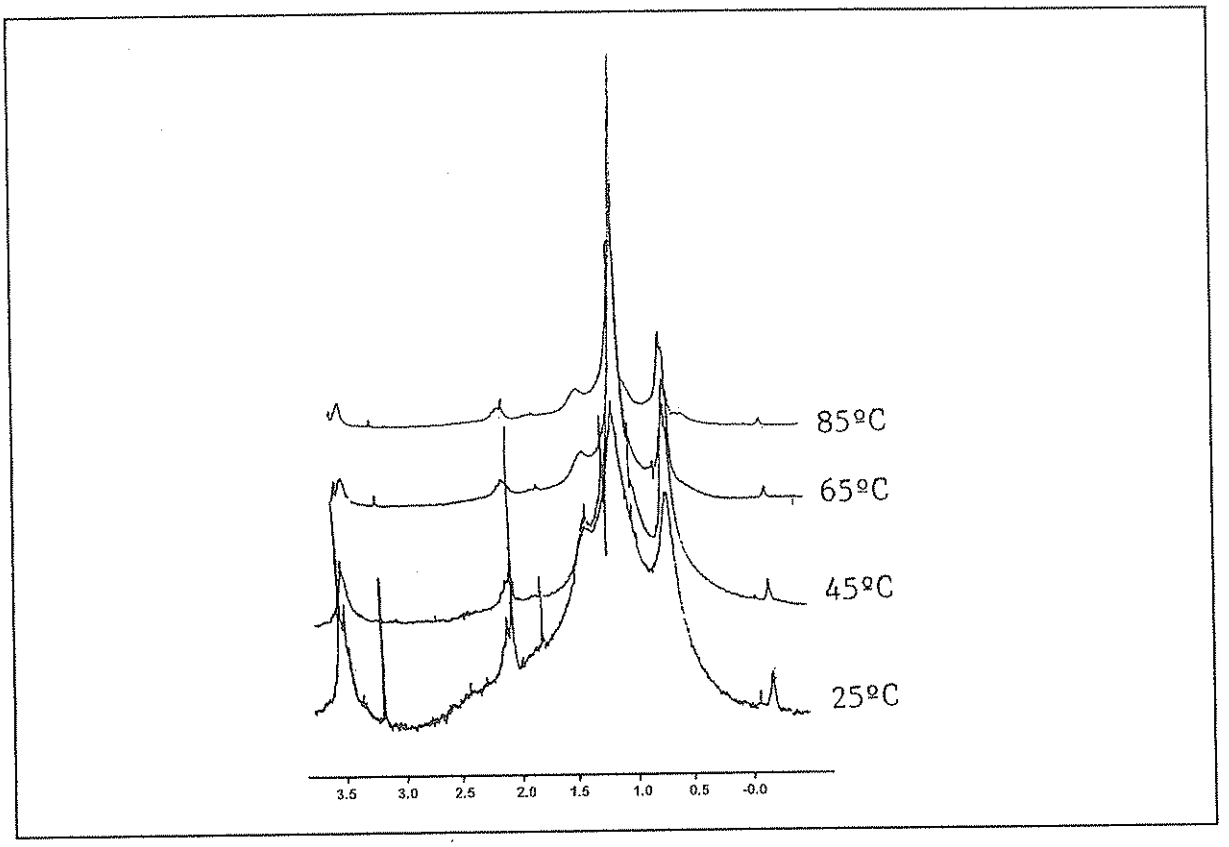

Fig. 1 - Temperature dependent ^1H NMR spectra of IWL vesicles in D_2O.

This value is much higher than the phase transition temperature of lipid mixtures usually used to form liposomes, such as phosphatidylcholine, below 0°C. However, it is 15 degrees lower than the value obtained for human horny layer lipids (75°C (6)). This difference could be due to the different lipid composition, especially a smaller amount of cholesterol in the IWL liposomes. *The particle size distribution* of the IWL liposome suspensions was determined at one and twenty-four hours after vesicle preparation. The results are expressed in Table II.

TABLE II - Particle size distribution, encapsulation efficiency and internal volume of IWL vesicles.

SAMPLES	PARTICLE SIZE DISTR. Average mean (nm)	Polydisp.	ENCAP.EFF. (%)	INTER.VOL. µL/mg
IWL 1h	265	0.173	0.076	0.4483
IWL 24h	282	0.276		

A particle size average mean distribution of about 270 nm and a polydispersity index around 0.2 was found at the two different times, achieving in both cases a size range of 150-500 nm. The stability of this suspension was shown to be longer than a week.
A smaller size range was found by other authors who formed liposomes with lipids from keratinized tissues; 20-150 nm with stratum corneum lipids [6], 20-90 nm with human hair lipids [8] and 20-80 nm with wool lipids [8]. The

greater size obtained in our case versus the results of other authors could be attributed to the lower temperature 65°C, and the shorter sonication time, 15 min, at which IWL liposomes have been formed.

The encapsulation efficiency and internal volume of the IWL vesicles (Table II) are, as in the case of SCL liposomes [18], much lower than those published for PC vesicles of similar size, irrespective of whether they are multilamellar vesicles (MLV) or large unilamellar vesicles (LUV) [19]. Surprisingly, the values of encapsulation efficiency and the internal volume corresponded to the values obtained for small unilamellar vesicles of PC with an internal diameter 10 times inferior to the one obtained for IWL liposomes.

Spontaneous permeability and transmission electron micrographs can shed light on the nature of the structural associations leading to the formation of different types of liposomes which may be correlated with the nature of the component building bilayers.

The bilayer permeability of the IWL liposome suspensions were followed by kinetic studies on the release of the fluorescent dye 5-(6)CF encapsulated in the interior of bilayers as a function of time in the absence of any surfactant. The results of this spontaneous permeability compared with those obtained from phosphatidylcholine (PC) and stratum corneum lipids (SC) presented elsewere [18] are expressed in Figure 2.

As in the case of stratum corneum lipids, the spontaneous permeability of IWL is extremely low when compared with the PC liposomes. This behaviour could suggest the higher permeability of the membranes of viable cells, mainly composed of phosphatidylcholine, with respect to the membranes from keratinized tissues such as stratum corneum or wool.

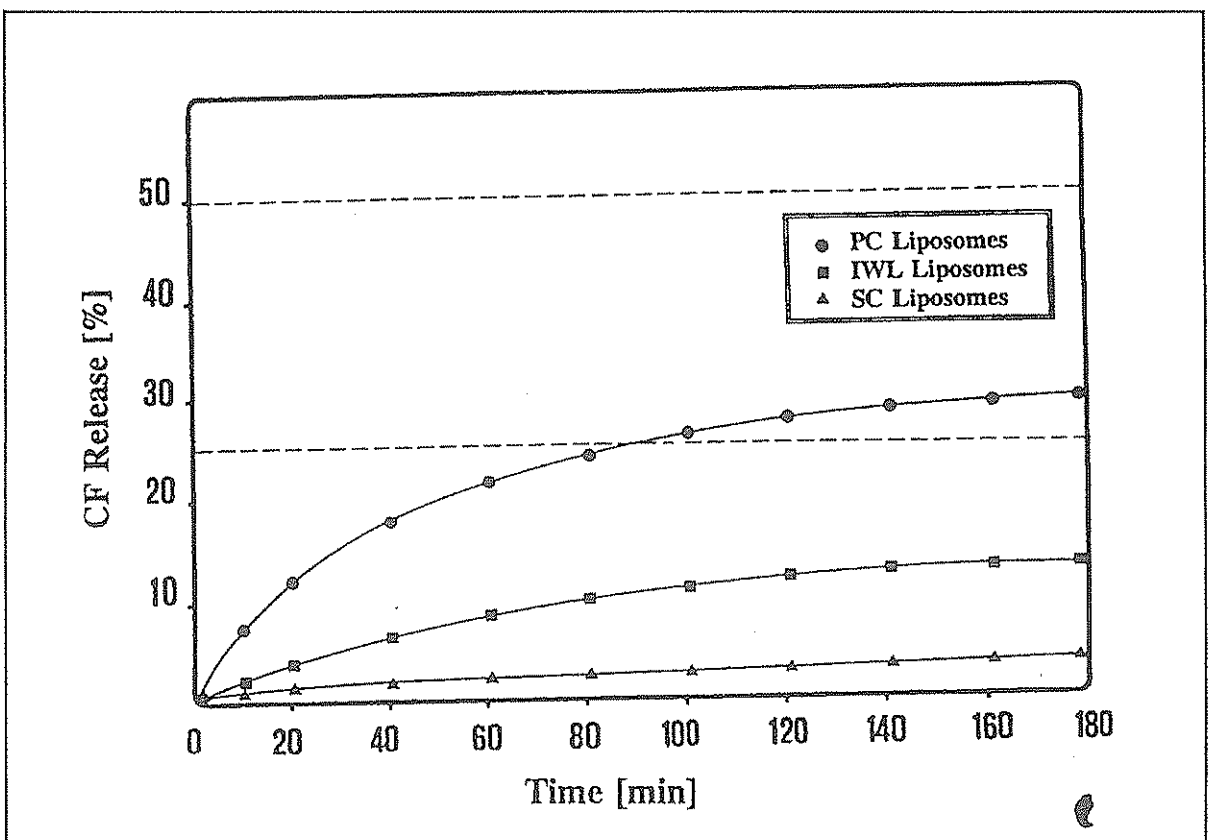

Fig.. 2 - Spontaneous permeability of liposome suspensions of IWL, phosphatidylcholine (PC), and stratum corneum lipids (SC) as a function of time.

TEM microphotographs of samples corresponding to the IWL liposome suspensions were examined using the TEM technique with freeze fracture and negative staining methodologies in order to determine the type of structures formed and to confirm the influence of the internal wool lipids on the liposome structure and vesicle size. Two representative pictures with freeze fracturing and negative staining are shown in Figures 3 and 4, respectively.

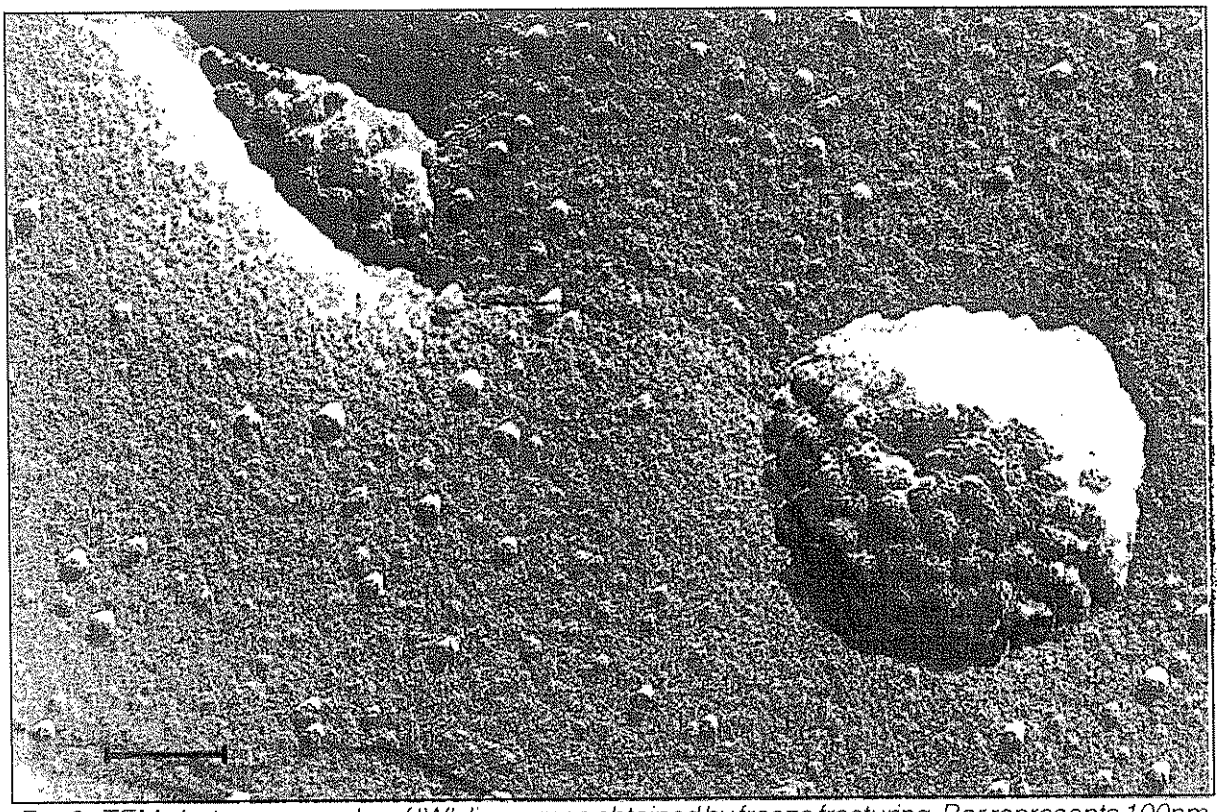

Fig. 3 - TEM photomicrographs of IWL liposomes obtained by freeze fracturing. Bar represents 100nm.

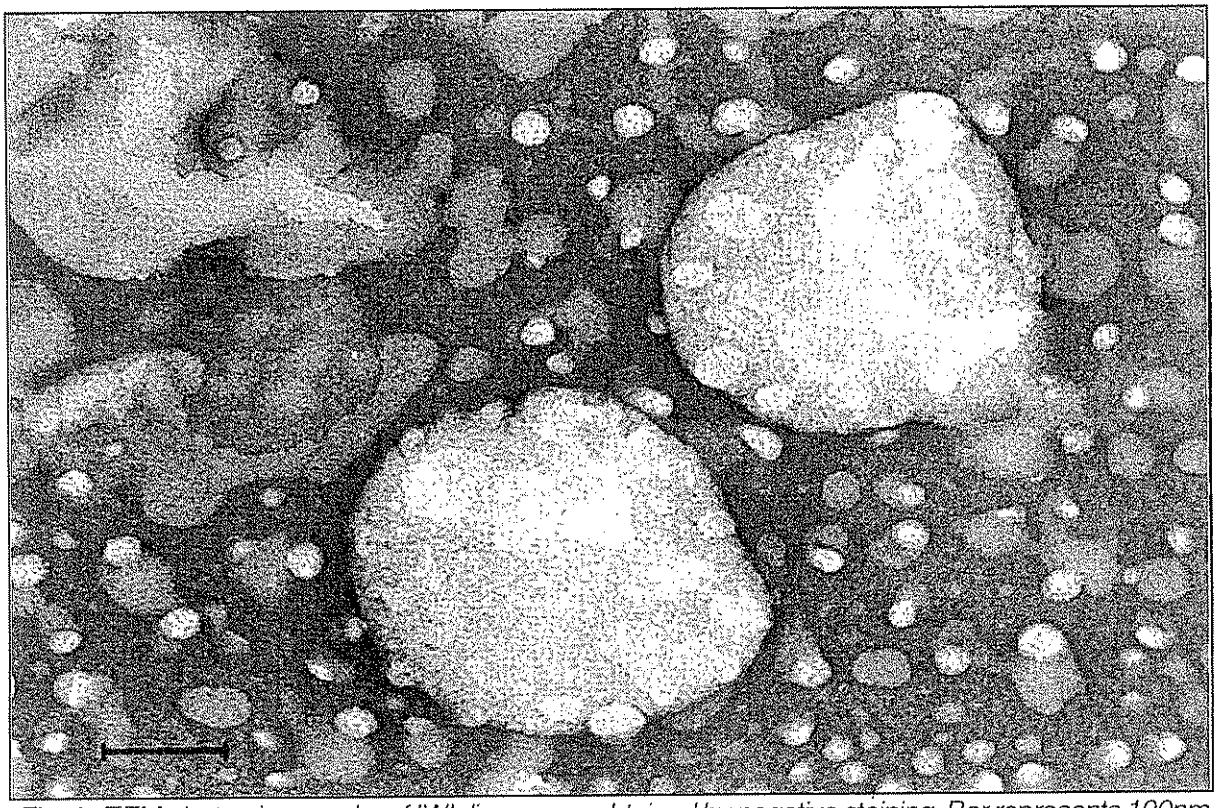

Fig. 4 - TEM photomicrographs of IWL liposomes obtained by negative staining. Bar represents 100nm.

There is an agreement with the microphotographs obtained by the two methodologies. There are big structures approximately 300 nm in size and much smaller structures approximately 20 nm in size. The most striking feature is the particular "strawberry like structure", more clearly shown in Figure 3. This seems to be made up of smaller size vesicles.
The controversial results previously discussed, on the one hand big vesicle size distribution and on the other hand a small internal volume, could be explained by this unusual structure in which small vesicles with a small internal diameter form bigger structures leading to a high size distribution.

CONCLUSIONS

Internal wool lipids were extracted and quantified. The lipid percentages obtained, sterol esters (10%), free fatty acids (24%), sterols (11%), ceramides (46%) and cholesterol sulfate (9%), were shown to form stable liposomes. A phase transition temperature of 60°C was obtained by NMR spectra performed from 25 to 90°C.
The spontaneous permeability of these vesicles was found to be very small, similar to vesicles formed with lipids from other keratinized tissues. A particle size average mean distribution of 270 nm was controversial given the low encapsulation efficiency and small internal volume. However, the TEM micrographs show big structures of approximately 300nm in size made up of smaller structures of approximately 20nm in size which could explain these results.

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REFERENCES

1. J.D. Leeder, Wool Science Review, 1986, 63, 3-35.
2. D.E. Rivett, Wool Science Review, 1991, 67, 1-15.
3. L. Coderch, C. Soriano, A. de la Maza, P. Erra and J.L. Parra, J.L., J. Am. Oil Chem. Soc. 1995, In Press.
4. A. Körner, H. Höcker and D.E. Rivett, Fresenius J. Anal. Chem., 1992, 344, 501-509.
5. P.M. Elias, Int. J. Dermatol., 1981, 20, 1-19.
6. P.W. Wertz, W. Abraham, L. Landmann and D.T. Downing, J. Invest. Dermatol., 1986, 87, 582-584.
7. D.T. Downing, J. Lipid Res., 1992, 33, 301-313.
8. A. Körner, S. Petrovic and H. Höcker, Textile Res. J., 1995, 65, 56-58.
9. A. de la Maza, A.M. Manich, L. Coderch, P. Bosch and J.L. Parra, Colloids and Surfaces, 1995, In Press.
10. J.N. Weinstein, E. Ralston, L.D. Leserman, R.D. Klausner, P. Dragsten, P. Henkart and R. Blumenthal, in: Liposome Technology, Ed. Gregoriadis G., CRC Press Inc., Boca Raton Florida, 1986, III, Chapter 13.

11. D.J. Gale, R.I. Logan and D.E. Rivett, Textile Res. J., 1987, 57, 539-542.
12. I.H. Leaver, D.M. Lewis and D.J. Westmoreland, Textile Res. J., 1988, 58, 593-600.
13. L. Coderch, C. Soriano, A. Pinazo, J.L. Parra and P. Erra, Textile Res. J., 1992, 62, 704-709.
14. L. Coderch and C. Soriano, Textile Res. J., 1993, 63, 369-370.
15. A. de la Maza, J.L. Parra and J. Sanchez Leal, Langmuir, 1992, 8, 2422-2426.
16. A. Schwan, J. Herrling and H. Zahn, Colloid Polym. Sci., 1986, 264, 171-175.
17. G.L. Scherphof, J. Damen and J. Wilschut Interaction of Liposomes with Plasma Proteins in: Liposome Technology, Ed. Gregoriadis G., CRC Press Inc., Boca Raton Florida, 1986, III, Chapter 14.
18. O. Lopez, A. de la Maza, L. Coderch and J.L. Parra, J. Am. Oil Chem. Soc., 1995, In press.
19. D.W. Deamer and P.S.Uster, Liposome Preparation: Methods and Mechanisms in "Liposomes" Ed. M.J. Ostro, Marcel Dekker, New York, 1983, pp 27-51.

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