Invited Review

Importance of interfacial water in permeabilization of ceramide bilayers

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Summary. The role of structured water at the membrane-aqueous interface in regulating the water permeability functions of the epidermal horny layer (Stratum corneum) is not clear at present. The subject of this review is the effect of perturbed interfacial water structure on the relative water permeability properties in shrinkage experiments of reconstituted vesicle suspensions of ceramides and cholesterol in the gel phase. Water structure was perturbed by a series of reagents known to affect the phase equilibrium of lipid assemblies. Multilamellar bilayers containing synthetic ceramides N-palmitoyl-D-L-dihydrosphingosine, and N-oleoyl-D-sphingosine were used as model membranes. The reagent binding to the ceramide membrane was determined by fluorescence measurements with 1-anilino, 8-naphtalene sulphonate (ANS). A dependence of number of binding sites for ANS on the type of reagent was found. The change of relative water permeability with concentration of reagents was studied. Analysis of these data reveals that the anomalous behaviour of relative water permeability with the concentration of reagents used is due to concentration-dependent structural transitions of the bound water at the membrane-aqueous interface. Different possibilities of interaction of the reagents with the interface are discussed. At the end, it is suggested that the interfacial water in multilamellar ceramide structures may also contribute to the water holding and permeability barrier functions of cell membrane complex within the stratum corneum.

Key words: Ceramides, Bilayers, Interfacial-water, Water structuring, Disordering reagents, Osmotic-shrinkage

Introduction

This review is primarily concerned with the effect of interfacial water on the passive permeability of ceramide

containing suspensions with multilamellar arrangement. It is known that the epidermal horny layer (stratum corneum) of mammals, birds, and reptiles contains multiple layers of lamellar membrane sheets occupying the intercellular space between the corneocytes. These membrane lamellae, which contribute significantly to the permeability barrier properties of skin, have an unusual lipid composition consisting mainly of ceramides, cholesterol, and free fatty acids. The ceramide component of stratum corneum is chemically heterogeneous due to the sphingosine and N-linked fatty acid moieties differing in chain length and unsaturation. The structural arrangement and mode of interaction of the lipid components of stratum corneum are not well understood. Associated with a protein-aqueous matrix, these lipids form the cell membrane complex, which serves as a "barrier" to the diffusion process in hair fibre (Hilterhaus et al., 1989). Moreover, they are regarded as an important determinant in water-retaining (Elias and Friend, 1975; Imokawa and Hattori, 1985; Imokawa et al., 1986), and water permeability barrier properties (Akasaki et al., 1988; Grubauer et al., 1989), by means of forming a multilamellar architecture within the intercellular spaces of stratum corneum. It has also been shown that ceramides may serve as a bound-water modulator through the formation of such ordered structures. This function of the ceramides is thought to be completely different from the water permeability barrier. Until now, a number of experiments on the water behaviour of ceramides have used an isolated powder of plantar stratum corneum. The isolation procedure needs a series of chemical treatments on the skin surface which can induce a marked damage to the lamellar structures of ceramide layers. Under these conditions the obtained stratum corneum lipid contains about 50% ceramide. On the other hand it was found that the synthetic ceramides exhibited water-retaining properties similar to those of the natural lipids. In such cases, vesicle suspensions containing a different class of ceramide may be successfully employed as models in studying the mechanisms of membrane permeabilization and water-holding processes within the lipid membrane. In practice, ceramides with relatively short non-branched,

and saturated alkyl chain are mainly associated with the water-holding function, whereas ceramides containing unsaturated fatty acid with longer alkyl chain serve as a permeability barrier. For this reason, the in vitro lamellae-forming ability of ceramides is closely related to their in vivo water holding capacity (Imokawa et al., 1989). In contrast, resistance against the water vapour flux reflects the water permeability barrier functions.

Although the biological importance of the ceramides, in epidermis and cellular processes, is well established, relatively little effort has been made towards elucidating the physico-chemical properties of ceramides. However, there is essentially no information concerning the behaviour of ceramide suspensions in the presence of substances known as water structuring and disordering agents at the membrane-water interface.

Nature of the available data

The role of large amounts of membrane-bound water in the lipid bilayer of stratum corneum in keeping skin supple and flexible is not clear at present. Based on data in literature, it can be shown that 0.13 mg of the ceramide isolated from stratum corneum sheet can hold nearly the same quantity of bound water (Imokawa and Kawai, 1991). The bound water can be supercooled (Hauser, 1975), behaves as nonsolvent water (Katz and Diamond, 1974) and possesses a high degree of order through water-water and water-lipid hydrogen bonding (Yeagle et al., 1975; Philips et al., 1982; Boggs, 1990). A high degree of water structuring has been observed at the polar headgroup region of some biological membranes (Cerbon, 1970). Permeability functions of these membranes to water and various solutes is however, known to be affected by the presence of an unstirred layer of water around the membrane (Naftalin, 1971; Jung et al., 1973; Cass and Finkelstein, 1976; Andreoli and Troutman, 1981). We have shown (Neitchev and Kostadinov, 1986) that alteration of water structure at the lipid-water interface in a bilayer by a series of reagents (also known to affect structuring of water molecules in bulk phase) influences the arrangement of membrane glycoproteins in phospholipid liposomes. Such alteration of the water structure in a liposomal membrane may be expected to affect its water holding and permeability functions. Although much research has recently been carried out on ceramides, their role in this phenomena has not yet been studied and should be of considerable interest in elucidation of one of the reasons for a loss of hair humidity in a pathologic skin as seen in atopic dermatitis or xerosis.

Choice of a model and preparation of samples

Here we used vesicle suspensions containing ceramide and cholesterol as a model of lipid bilayers in stratum corneum. Cholesterol is also a relatively abundant component of stratum corneum and has been proposed to affect its permeability properties. The effect of altered water structure at the lipid-aqueous interface on the osmotic shrinkage properties of vesicles was investigated as a function of unsaturation and chain length of N-linked fatty acid moieties. However, lipid mixtures in which palmitic or stearic acids were replaced by oleic acid did not form lamellar structures. This indicates that the stable bilayer formation of the stratum corneum is strongly dependent on the molecular fatty acid structure.

The present study was performed by using N-palmitoyl-D-L-dihidrosphingosine (PDHS) and N-oleoyl-D-sphingosine (OS) purchased from Sigma (Ref. P9635 and Ref. O7378). They were given as a gift from the Center of L,Oreal in Tsukuba, Japan and were used without further purification. Cholesterol and fluorescence markers were obtained from E. Merck (Darmstadt, Germany).

Water structuring and disordering agents were commercial products (Sigma) of analytical grade. A series of water soluble amines (methylamine, ethanolamine, hydrohylamine, ethylendiamine and triethanolamine) were used as structuring agents. Urea, thiourea, dimethylsulfoxide (DMSO), formamide and $NaClO_4$ were used as disordering agents. 1M solutions of these reagents (except for $NaClO_4$) were prepared in 10 mM Tris-HCl and pH was adjusted to 7.4.

Required amounts of ceramide and cholesterol (weight ratio 50:15) in chloroform solution were taken in flat-bottomed glass vials and in a thin film under nitrogen and vacuum. Solutions with different concentrations of structuring reagents, cited above, were saturated with nitrogen and then added to the individual vials to make the final ceramide concentration about 1-2 μM/ml. After sealing the vials under nitrogen, the mixture was dispersed by intensive swirling using several small metal balls with diameter of about 2-3 mm placed in the vials. The mixture was vortexed 4-5 times for 1-3 min at 95 °C and the resulting suspension was allowed to equilibrate at room temperature for 30 min. and then rapidly cooled down to about 5 °C. The heating and cooling processes were repeated several times. The mixture of ceramide and different reagents was placed in a cylindrical bath sonicator for 10 minutes at 95 °C until it formed a homogenous emulsion, then centrifuged at 12000g for 15 min, before its use in osmotic shrinkage experiments. The samples thus obtained were assayed for ceramide content by photodensitometry (Shimazu CS-9000). All ceramides were quantified by determining the weight of ceramides in mg/ml of suspension on a thin-layer chromatogram chart as described previously (Yoshikawa et al., 1994).

Vesicle size and characterization

Previous investigators had indicated that difficulties exist in dispersing synthetic ceramides in water (Braida et al., 1994). The problems that were encountered may in part be due to the use of mixtures of hydroxyceramides and ceramides without hydroxy fatty acids, because the

latter do not appear to undergo well-defined transitions or to form a lamellar phase. Due to the metastability of mixtures of ceramides and cholesterol, variable conditions for preparation of vesicle suspension were used. Suspensions produced by the procedure described above yielded a relatively homogeneous population of middle-sized vesicles. The ceramide analysis of supernatant resulted in recovery of 25-30% of total ceramide as multilamellar vesicles. For more precise observation of these particles, an electron microscopy study was applied. A small volume of vesicle suspension was fixed in 2.5% glutaraldehyde in the same buffer, as above for 2 h and postfixed with 0.2% ruthenium tetroxide (Sigma) for 1 h at room temperature. After dehydration with propylene oxide, thin sections of the sample were stained with lead citrate, and observed by a Jeol 100B apparatus at 80 kV.

The particle size distribution was determined by a Coulter Electronics, particle analyzer Hialeah, Fl. Suspensions obtained by the procedure described above yielded a relatively homogeneous population of middle sized vesicles as determined by negative stain electron microscopy studies (Fig. 1). Morphologically, the vesicles were spheroids with granular internal structure and were very similar to those described in literature (Kim, 1983) and named "multivesicular" particles. A bilayer formed the outer-most membrane and the internal space was divided up into numerous compartments by bilayer septums. The data received from the particle analyser are plotted in Fig. 2. The results showed approximately a Gaussian size distribution for both, PDHS (Fig. 2A) and OS (Fig. 2B) particles. The maximum of particle size distribution was at 65 nm for PDHS, and at 85 nm for OS containing suspensions. The OS suspensions contained larger particles which is reasonable since these samples were sonicated for shorter time. The difference in the size of distribution may also be due to different fatty acid composition. Analysis of the fatty acid composition has revealed that ceramides containing unsaturated fatty acid chains are better dispersed in water (Shah et al., 1995). Therefore, such suspensions may form spontaneously-sealed vesicles with larger diameter. The mean diameter of these particles was also larger. The widths of corresponding distributions were nearly 50 and 25 nm. The average size distribution became narrower as the mean diameters decreased. The PDHS vesicles seemed to be more homogeneous particles.

Osmotic shrinkage experiments

All data related to water permeability of lipid vesicles indicate that multilamellar particles can be used successfully to determine the outflow of water (in bulk phase also) under passive transport conditions. The modules of elasticity of such vesicles are sufficiently high, so that even small changes in the volume will lead to a loss of internal water content which would slow down the increase in vesicular volume during dilution of medium (Rickey and Williams, 1990). The literature is
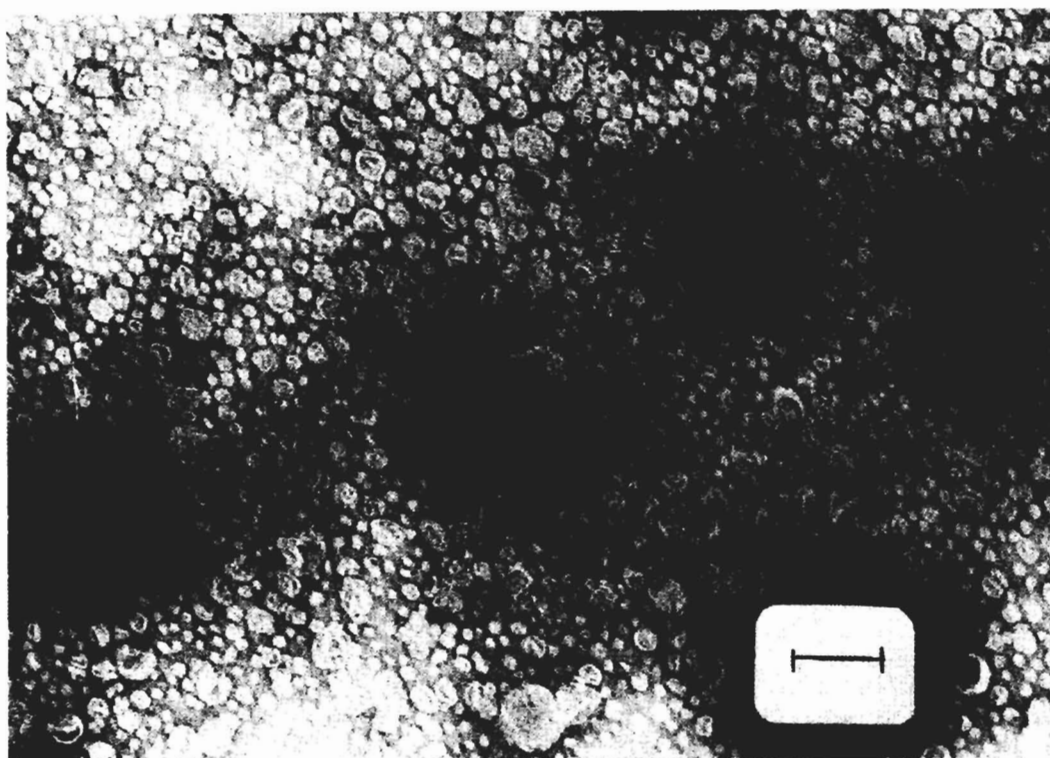

Fig. 1. Electron microscopy measurements of vesicles containing PDHS with final ceramide concentration of 0.67 mg/ml in 10 mM Tris HCl buffer, pH 7.5. The suspension is incubated for 2 hr and then left at room temperature for approximately 1 hr before fixation with OsO_4 Bar: 150 nm.

extensive and cannot be covered in this short review. However, a limited discussion of this matter is included because some of the findings are directly relevant to osmotic shrinkage of lipid vesicles. When volume changes of vesicle suspensions are followed via changes in static light scattering, the data are sensitive to the morphology and dispersivity of the samples (Ertel et al., 1993; Hallet et al., 1993; Mui et al., 1993). The in-homogeneity is a less important factor in the fast shrinkage phase. The problem has been approached both experimentally and theoretically, and it was shown that the approximation of Rayleigh-Gans can be extended to a number of particles such as native and artificial membranes with diameter from 0.01 to 1 μm (Koch, 1968; Latimer et al., 1968; Latimer, 1983). Starting from this approximation, light-scattering is characterized by the quantity (Chong and Colbow, 1976).

$$S = 24\pi^3 V^2 N \, (n/\lambda^4 \, (m^2 - 1/m^2 + 1)^2 \, FQ \quad (1)$$

or $S = h.F.Q$. Here, V is the volume of tested solution, N the number of particles in the solution, n the refractive index of medium, $m = n_0/n$ the relative refractive index, where n_0 is the refractive index of the particle, F the incident intensity, and Q the dissipation factor that accounts for the interference of light-scattering from different parts of particle, F and Q being described in detail (Chong and Colbow, 1976). The function h is independent of the vesicle properties. A simple way to relate S to the change in vesicular radius r is by taking the logarithmic form of (1) and then differentiating it. It has been shown previously that F is fairly insensitive to changes in r (Chong and Colbow, 1976). In our studies, the shrinkage experiments were done at 480 nm, and the volume dependence at 900 scattered-light intensity could be estimated from the equation valid for vesicles:

$$d\ln S/d\ln V_0 = 0.67 \quad (2)$$

where V_0 is the molar volume of the water inside the vesicles (Yoshika et al., 1983).

Osmotic shrinkage properties of samples were studied as described in previous work (Neitchev et al., 1997). Efflux of water content from vesicles was monitored as an increase in fluorescence of 175 mM calcein. Osmosensitivity measurements were performed after addition of sucrose solution with concentration from 0.5 to 1 M to a suspension of these samples. The last caused a nearly 15% increase in the light scattering intensity. The reciprocal of the maximum changes of light-scattering (1/Imax) are plotted against the reciprocal of the sucrose concentration difference across the membrane (1/Δc). As predicted by the Boyle-van't Hoff equation a linear relationship was observed between the two variables.

Osmotic shrinkage of samples was observed with a Durrum stopped-flow apparatus The light source was a Spectrophysics 165Ar$^+$ laser. Two injection syringes were used. The first contained a suspension of samples

with added reagents in the same buffer as described above. The second was filled with a hyperosmotic solution of sucrose at an hyperosmotic concentration of about 300 mosmol in addition to the buffer used. The time dependence of light-scattering intensity, I (mV) was followed at room temperature. The initial shrinkage velocity was then defined as:

$$V_0 = - (dI/dt)/I^2 \quad (1)$$

V_0 is proportional to the rate of outflow of vesicular-bound water, which corresponds to the relative water permeability P_{rel} and can be expressed by the relation:

$$P_{rel} = V_0^{sample} / V_0^{control} \quad (2)$$

where $V_0^{control}$ is related to the to pure suspension without water structuring and disordering agents. All stopped flow experiments were carried out independently at least 3 times. The signals were amplified, then visualized on the monitor of an Apple computer and stored with an appropriate time base. To follow slow volume changes a Perkin Elmer 3000 dual path absorption spectrophotometer with a 1 cm square cell was used.

Determination of the reagent binding

The fluorescence marker ANS that binds to the lipid membrane was used to monitor the binding of water structuring and disordering reagents at the membrane-aqueous interface. This substance has been used to study the changes in protein conformation, as the fluorescence properties of ANS depend upon the polarity of its environment. It becomes fluorescent when it is stochiometrically bound to sites in the hydrophobic regions at the lipid-water interface. Through the dye

Table 1. Mean values of number of binding sites (n) for ANS in pure suspension of free vesicles of ceramides (control) and such containing different water structuring and disordering reagents. Values are given ± SD, from 3 separate experiments (n=3) with their 95% confidence limits.

Species	n x 10² (mmol/mol ceramide)	
	PDHS	OS
Pure suspension	3.1±0.11	3.8±0.28
Methylamine	6.2±0.24	7.4±0.32
Hydrohylamine	7.1±0.26	7.9±0.33
Ethanolamine	6.4±0.27	7.1±0.26
Ethylenediamine	6.9±0.38	7.7±0.26
Triethanolamine	7.5±0.28	8.7±0.49
Urea	2.5±0.21	3.8±0.25
Thiourea	2.6±0.20	3.1±0.20
Dimethylsulfoxide	1.3±0.08	2.7±0.16
Formamide	0.9±0.07	1.8±0.10
Sodiumpercholorate	4.1±0.37	4.5±0.38

The concentration of all reagents was 0.4 M, with the exception of sodiumperchlorate, equal to 4 mM.

binding studies, our aim was to find whether direct binding of the reagents to the vesicle membrane was responsible for the structural order at the membrane-aqueous interface. Binding studies were performed as described in previous works (Das and Singhai, 1981; Neitchev, 1984). ANS with concentration of about 40 μM was added to the stock suspension of vesicles containing different reagents at initial concentrations of 0.4M. After 30 min of incubation at room temperature the flourescence was measured in a Perkin-Elmer MPF-4 spectrofluorimeter with temperature control and recorder attached. Excitation was at 380 nm. Fluorescence intensities were measured at the maximum emission wavelength, 460 nm. The observed fluorescence intensities were corrected first for the emission of the pure suspension and of free ANS, then for the absorption of different samples, as given in Table 1. From the double reciprocal plots of fluorescence intensities against ceramide or reagent concentrations in the samples at fixed ANS concentration, it was concluded that these fluorescence changes can be attributed to changes in the binding parameters of ANS to vesicles and not to changes in the quantum yield of bound ANS. The number of binding sites n may be calculated from the equation:

$$ANS_{add} / F = 1/B + K_d / B \times n (P_i) \quad (3)$$

where F is the measured fluorescence intensity, ANS_{add} the added ANS concentration, B a constant relating the fluorescence intensity and P_i the ceramide concentration. When ANS_{add} is greater than the ceramide concentration n may be determined using a plot of the 1/F against P_i. K_d is calculated from the plot of 1/F versus 1/ ANS_{add} as shown in literature (Okuda, 1982). The latter always yielded a linear ralationship. Table 1 shows experimental values for the number of binding sites n, for free vesicles (control) and vesicles incubated with solution of reagents used. An increase of n was observed, against the n values for the free vesicles, in all cases with amine
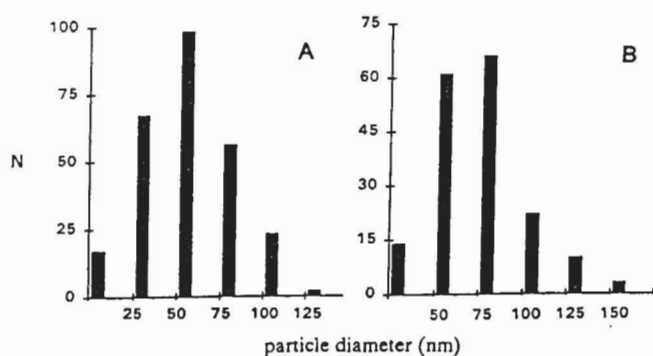

Fig. 2. Vesicle size distribution. N is the number of counted particles. **A.** Vesicles with PDHS. **B.** Vesicles with OS. The final ceramide content in the supernatant is about 0.7 mg/ml in both cases. Mean values ± SE for vesicle diameters are: A) 60±28 nm (n=12); and B) 74±32 nm (n=166). The values are derived from 95% confidence limits.

containing reagents. It seems likely that the increase of n in these vesicles are caused by the increase of hydrophobic regions in the membrane. In contrast to amine containing reagents, all other substances listed in Table 1 showed a decrease of n values in comparison with free vesicles (except for $NaClO_4$). The last result can be interpreted as a reduction of hydrophobic regions at the lipid-water interface. In the case of OS vesicles, the n values exceeded those for PDHS vesicles. However, the results of dye-binding studies reveal that none of the substances used as water structuring and disordering reagents bind directly to the vesicle surface. Perhaps, they influence the phase equilibrium of the lipid membrane by perturbing the structured water at the membrane-aqueous interface (Das and Singhai, 1981).

Analysis of permeability data in presence of water structuring reagents

The osmotic shrinkage of ceramide vesicles, after mixing with hyperosmotic solution of sucrose, is completed within a few minutes. Plots of initial shrinkage velocity V_o (see equation 1) against sucrose concentration were found to be linear in both PDHS and OS vesicles (data not shown), demonstrating that these vesicles behave as nearly perfect osmometers even with the highest concentration of reagents used (1.0M). The difference in the vesicle size had no effect on the rate of water outflow.

A noticeable feature of the dependence of relative water permeability P_{rel} (Fig. 3) on the concentration of reagents used is its anomalous behaviour in most cases. Each curve with a specific symbol represents P_{rel} obtained experimentally at various concentrations of the same reagents.

Amines have a multitude of possible reactions with water lattice (Jeffrey et al., 1967). The amino group of the molecule serves an important role as a proton donor on its interaction with water (Glasel, 1970). We examined the changes of P_{rel} in both cases, with PDHS vesicles and with OS vesicles. A decrease of P_{rel} was observed against the control values (without reagent) in the first case with various reagents. In the presence of amines, P_{rel} follows the same trend, as the concentration is changed increasing slowly from 0.2 to 0.6M and then decreasing. Marginal lowering of cooperativity of transition in PDHS vesicles in the presence of some amines takes place in the same concentration range where slow increase of P_{rel} was observed (Neitchev, private results). In contrast to these studies, in the case of OS vesicles the variations of P_{rel} in the same concentration range are opposite. The values of P_{rel} against the control are higher in most cases. There is a clearly expressed trend to an increase of P_{rel} to 0.6M accompanied by a decrease after this concentration. We have observed that the dependence of cooperativity of transition of PDHS and OS vesicles on concentration of these amines has nearly the same shape suggesting possible correlation of P_{rel} in both cases. Although much

research has recently been carried out on water permeability in natural and artificial phospholipid membranes, far less attention has been given to the same problem in ceramide containing membranes. From the data received in our study it is difficult to interpret the individual behaviour of these amines in the process of osmotic shrinkage in ceramide-containing liposomes. It is known that hydrohylamine and ethylendiamine have very similar effects on the membrane permeability of phospholipid vesicles. At higher concentrations (0.1 to 1M) they affect the size distribution and probability of formation of transient, statistical aqueous pores in the membrane (Blok et al., 1975, 1976). In the presence of ethylendiamine, P_{rel} of water and the extent of shrinkage exhibit concentration dependencies similar to those with hydrohylamine, suggesting the same action of these two reagents on the ceramide membrane. The similarity in

Fig. 3. Dependence of relative permeability of water, P_{rel} of PDHS and OS vesicles on concentration of water structuring reagents. Each data point is the mean of at least three experiments. Error limit being + 8.5%. Methylamine (-◊-◊), ethylendiamine (-□-□), hydrohylamine(-Δ-Δ), ethanolamine (x-x), trhriethanolamine (-*-*).

their action on osmotic shrinkage properties in ceramide-containing vesicles is interesting, in view of the presence of multiple functional groups on these molecules. The notable similarity between variations of P_{rel} on the presence of ethanolamine and triethanol-amine may be due to their molecular similarity.

We proceed to discuss the anomalous behaviour of permeability data in this study. The reduction of P_{rel} after a concentration of 0.6M may be due to inter-calation of the bulky amine molecules between the polar headgroups interfering with hydrogen bonding patterns and van der Wall's interactions. At higher concentrations of amines, the extent of shrinkage decreased slightly thus indicating an increase of structural order at the membrane-aqueous interface. The last is connected with a gradual increase in the size of structured water clusters combined with molecules of amines.

The difference between the values of P_{rel}, against the values of controls in both cases, showed that OS vesicles are more permeable to the outflow of internal vesicle water, than those of PDHS. This is in agreement with the classic finding that lamellar structures of ceramides with long and unsaturated alkyl chains demonstrate water permeability functions. The sharp reduction of P_{rel} after a concentration of 0.6M in this case may be due to steric factors derived from the double bonds of unsaturated alkyl chains. The process of formation of water clusters in such a case is more obvious.

Analysis of permeability data in presence of water disordering reagents

Plots of P_{rel} against the concentration of these reagents is given in Fig. 4. An increase of P_{rel} was observed in both cases. In the presence of urea and thiourea, the structuring of the vesicle-entrapped bulk water is presumably higher yielding minimal quantity of free water due to the action of these reagents. The difference between the curves in the presence of urea and thiourea reveals the importance of functional groups in determining the potency for modulation of water structure at the membrane-aqueous interface.

Ice-like water clusters are known to be formed in the presence of DMSO (Szmart, 1985). Formation of such clusters at the membrane-aqueous interface and both sides of the lipid membrane may increase the total amount of internal volume available for shrinking and may also be responsible for an increase of V_0 of the sample. At higher concentrations DMSO probably replaces some water-structured molecules from the polar headgroup region and takes part in hydrogen bonding (Lyman and Papahadjopoulos, 1976; Lyman et al., 1976; Szmart, 1985), causing an increase in the extent of shrinkage. It may be noted that the biophysical activity of several small molecules, e.g. DMSO, urea and t-butanol in promoting denaturation is known to depend upon their competing interaction with the interface or substrate for water molecules and functional groups
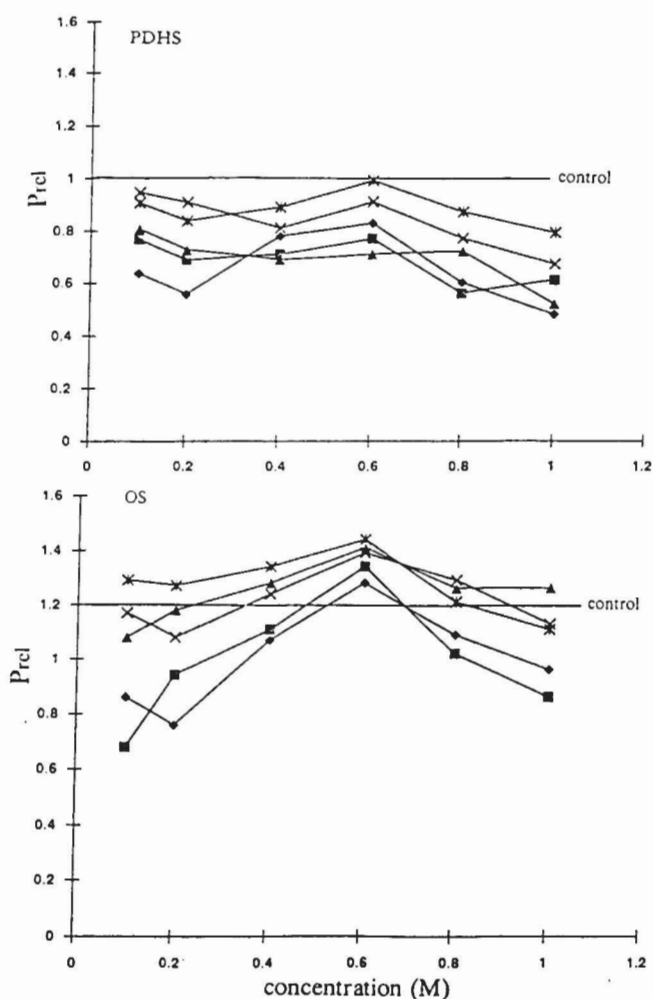
present in the molecule (Glasel, 1970). In the presence of DMSO at all concentrations higher than 0.2M, P_{rel} is higher compared to the control (Fig. 4). This is probably due to passage of water through transient statistical pores owing to increased size of the clusters, as well as restricted rearrangement of hydrogen bonds in the polar headgroup region (Oku et al., 1980).The presence of formamide in the vesicles shows a similar trend to that described above.

The addition of $NaClO_4$ with a concentration of 0.1M to the vesicle suspension of ceramides induces a precipitation predominantly in the crystalline state. It is well known that ClO_4^--ions have a very strong structure-breaking tendency to perturb the ordered lipid structures (Pigor and Lawaczek, 1983). Measurements on the water outflow in shrinkage experiments of such vesicles as function of the concentration of $NaClO_4$ failed above 10 mM of this reagent. The variation of the $NaClO_4$ concentration revealed that a threshold concentration of $NaClO_4$ in the range from 1 to 10 mM induces changes in the initial shrinkage velocity. Above this concentration precipi-tation becomes visible in the time range studied (minutes). Fig. 5 shows the dependence of P_{rel} on the concentration of $NaClO_4$ in the cases with PDHS- and OS-containing vesicles. There is a close similarity between curves 1 and 2 in both cases. The corresponding values of P_{rel} are higher than the controls. This is probably due to an increase of water passage through transient statistical pores that accompany structural defects in the membrane created by $NaClO_4$ interacting with the interface. It can be seen that higher $NaClO_4$ concentrations induced significant sharp enhancement of P_{rel}, up to a concentration of about 4 mM. Above this concentration a decrease of P_{rel} was observed. The latter is due to an increase in the vesicle aggregation at higher concentrations of ClO_4^- ions (Pigor and Lawaczek, 1983). Thus it may be asked whether this $NaClO_4$ effect on vesicle shrinkage remains at the stage of aggregation via disordering of the membrane-aqueous interface. The dependence of n on the concentration of $NaClO_4$ (data not shown) showed a significant increase of n values against the control (pure vesicles) at concentrations higher than 4 mM. This may be due to formation of aggregated particles which is accompanied by a decrease of the hydrophilic regions at the membrane-aqueous interface. In agreement with the results given above, the values of P_{rel} in the case of OS vesicles are higher than those for PDHS. This effect is possibly associated with the previous finding that structural characteristics in alkyl chains are required for water permeability capacity in lamellar formations of ceramides (Imokawa et al., 1989).

This review provides the first experimental evidence that some substances known as water structuring and disordering reagents at the membrane-aqueous interface may influence the water permeability functions of ceramide suspensions in osmotic shrinkage experiments. Primarily three distinct possibilities of interaction may be vizualised in the system studied by

us: ceramide-solute, water-solute and ceramide-water. The fluorescence binding studies clearly negate any direct ceramide-solute interaction. Water-solute interaction will conceivably modify the intrinsic structuring of bulk water thus influencing its anomalous properties and its fitness to act as a molecular environment (Klotz, 1970). Depending on the solute, it may facilitate formation of water clusters, or structures like water monomers. Water-solute interaction is known to depend on the finite molecular size of the solute and on its functional group (Franks, 1966). Such water-solute interaction may lead to a change in the size of water clusters in bulk water. The latter is of particular significance, considering the fact that osmotic pores in a lipid membrane are known to allow the passage of molecules of diameter less than 4 Å (Solomon, 1982). The water molecules near an interface are oriented by
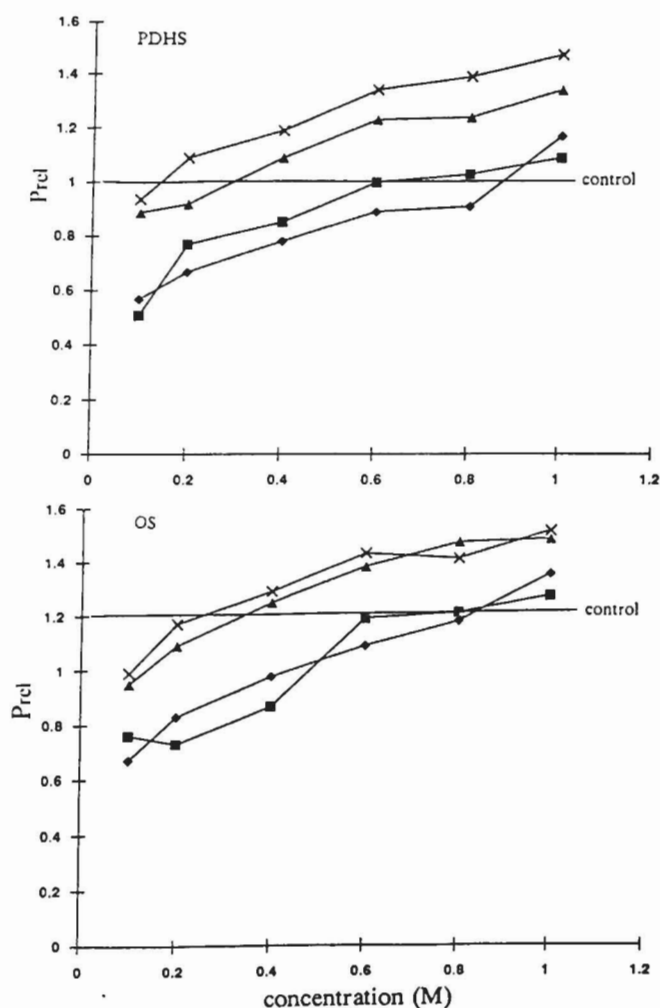

Fig. 4. Dependence of relative permeability of water, p_{rel} of PDHS and OS vesicles on concentration of water disordering reagents. each data point is the mean of at least three experiments. Error limiting + 8.5%. Thiurea (-◊-◊), urea (-□-□), dimethylsulfoxide (-Δ-Δ), formamide (x-x).

Interfacial water in ceramide bilayers

dipole-dipole interactions. It is known that the membrane-aqueous interface may stabilize the structured bulk water by acting mainly as a momentum sink for thermal fluctuations which may otherwise disrupt the lattice stability latently present in bulk water (Hauser, 1975). The water-solute interaction is thus expected to be reflected in lipid-water interaction. The bound water in a lipid membrane behaves as unfreezable and nonsolvent (Katz and Diamond, 1974). Different studies (NMR, DSC, Infrared spectroscopy and dielectric relaxation method) strongly point to the existence of highly ordered membrane-associated water. The last may pass from one form to another. This was confirmed in studies of thermal anomalies in various membrane properties such as membrane resistance, thermal transition in ultrathin BLM, and membrane potential of "Valonia ultricularis" (Thompson, 1964; Nelson and Blei, 1966; Hauser, 1975, 1976). It is conceivable that any agent which can modulate intrinsic structuring of bulk water and bring about structural transitions of ordered water at the membrane-aqueous interface would induce similar anomalies in membrane properties. We thus argue that the anomalous P_{rel} data shown in Figures 3-5 reflect a similar effect of these agents on the structured water at the membrane-aqueous interface, which induces concentration-dependent order-disorder transitions. Such transitions may also be responsible for the water permeability properties of ceramide vesicles, due to mutual interaction and stabilization of water and lipid bilayer. The careful consideration of available permeability and binding site data leads us to the conclusion that this is the most plausible interpretation.

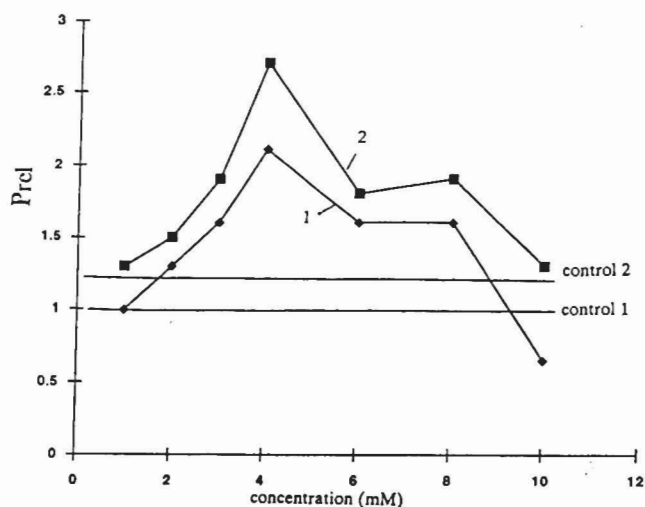The reagents used in this study may have various modes of action on the interfacial structured water. The vesicle membrane may be partially dehydrated and may become more rigid due to the competition between these reagents and lipid polar head groups for binding to water molecules or due to a cosolvent action of these reagents at a higher concentration where the reagent molecules may physically replace the water molecules from the interface.

In Figure 6 we have plotted P_{rel} values against their corresponding n values, as listed in Table 1 with the exception of $NaClO_4$. It is surprising that the data points deviate so markedly from the least square straight line. A close inspection of Figure 6 reveals that the reagents, which lie in the first group (water disordering reagents), may perturb the water structured order accompanied by an increase of P_{rel} at low n values, or by a decrease of hydrophobic regions at membrane-aqueous interface. The reagents in the second group (water structuring reagents) showed lower values of P_{rel} (except for some points) at higher n values, or an increase of hydrophobic regions. In this case, membrane dehydration leads to a decrease of membrane fluidity (Schneider et al., 1979), which in turn decreases membrane permeability.

The above discussion may be summarized as follows: our fluorescent dye binding data demonstrate that the reagents used to perturb the structured water in bilayers of ceramide vesicles are capable of influencing the phase equilibrium, without binding directly to the membrane, by altering the water structure at the membrane-aqueous interface. The latter leads to a change of P_{rel} in osmotic shrinkage experiments of vesicle suspension containing PDHS and OS as synthetic ceramides. In the case of water soluble amines considered as water structuring agents, the water permeability data showed anomalous concentration behaviour. On the contrary, when chaotropic reagents were used as water disordering factor, the water
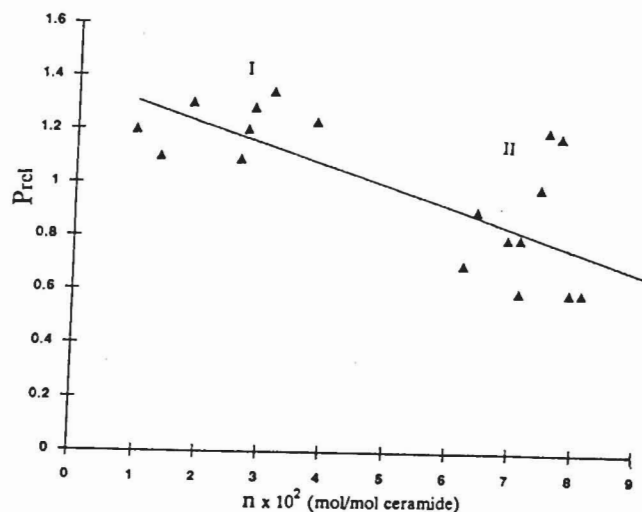

Fig. 5. Dependence of relative permeability of water, P_{rel} of PDHS (curve 1) and OS (curve 2) vesicles on cocentration of $NaClO_4$. Each data point is the mean of at least three experiments. Error limiting being + 10%.

Fig. 6. Observed apparent correlation between the relative permeability of water, P_{rel} and number of binding sites (n) for ANS as listed in Table 1. The concentration of all reagents was 0.4M. The slope of the straight line is determined with a correlation coefficient of 0.85 + 0.04 (n=18).

permeability in such vesicles increased significantly. Urea and thiourea showed lower values of P_{rel} as compared to other chaotropic agents. This evidence suggests that these substances are primarily responsible for the water-holding properties of ceramide vesicles. For this reason they have been termed as natural moisturizing factors. For both water-structuring and water disordering reagents the corresponding P_{rel} data, in the case of OS vesicles, were slightly higher in comparison with PDHS-containing vesicles. The last is in agreement with our finding about the role of the alkyl chain structure in determining the hydration behaviour of ceramide-containing bilayers.

Enhancement or disruption of intrinsic structuring of the entrapped bulk water in multiconcentric lamellae structures of ceramides by the reagents cited above probably contributes potentially to the their role in the water holding property of Stratum corneum. This may be a crucial factor in keeping skin supple and flexible. For this reason it is important from molecular point of view to know the effect of such reagents used as components in different hair care products.

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