A New Computer-Based Evaporimeter System for Rapid and Precise Measurements of Water Diffusion Through Stratum Corneum *In Vitro*

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It is important to have reliable methods for evaluation of skin barrier function when questions such as barrier perturbing effects of different agents and occlusive effects of different formulations are to be elucidated. A wealth of clinical work relates to measurements of transepidermal water loss *in vivo*, a method much affected by ambient air relative humidity, temperature, skin irritation processes, psychologic status of the subject, etc., factors that cause the method to suffer from low precision (i.e., high random error). Relating to these obstacles, we have developed a closed *in vitro* system for measurements of water diffusion rate through pieces of isolated stratum corneum at steady-state conditions, where the relative humidity and temperature is held constant and data can be collected continuously. Our evaporimeter-based *in vitro* system has a more than 3-fold higher precision (lower random error) (≈10%) than measurements of transepidermal water loss *in vivo* (≈35%). The results of our study show that: (i) the corneocyte envelopes contribute to the barrier capacity of stratum corneum; (ii) removal of the lipid intercellular matrix results in approximately a 3-fold increase in the water diffusion rate through the isolated stratum corneum ($n = 20$; $p < 0.05$), not a 100-fold as has previously been suggested; (iii) exposure to sodium dodecyl sulfate in water does neither alter the water diffusion rate ($n = 10$; $p > 0.05$) nor the water holding capacity ($n = 10$; $p > 0.05$) of stratum corneum; (iv) exposure to 1 M $CaCl_2$ in water yields an increased water diffusion rate through stratum corneum ($n = 10$; $p < 0.05$); and (v) when applied to the stratum corneum in excess concentrations, the penetration enhancer Azone has occlusive effects on water diffusion through the stratum corneum ($n = 6$; $p < 0.05$). ***Key words: azone/occlusion/oleic acid/SDS/skin penetration enhancers/surfactants. J Invest Dermatol 113:533–540, 1999***

When questions such as barrier perturbing effects of different agents and occlusive effects of different formulations are to be elucidated it is of great importance to have reliable methods for evaluation of skin barrier function. One of the simplest and most direct ways to evaluate barrier function is to measure the perspiratio insensibilis *in vivo* (e.g., by the evaporimeter technique). Transepidermal water loss (TEWL) measurements, however, are much affected by the ambient air relative humidity, temperature, skin irritation processes, psychologic status of the subject, etc., factors that cause the method to suffer from low precision (i.e., high random error) (Wilson and Maibach, 1994). Accordingly, to have any real use of this very simple and fast method (e.g., measuring the occlusive effects of different formulations and the barrier perturbing effects of toxic agents and solvents) these external conditions must be carefully controlled and standardized. Relating to these obstacles, we have constructed a closed *in vitro* system for measurements of the water diffusion rate through pieces of isolated stratum corneum (SC), where the relative humidity and temperature is held constant and data can be collected continuously and at steady-state conditions.

The barrier towards water loss is primarily located to the intercellular domains of the SC which are filled with stacked lipid bilayers (Breathnach *et al*, 1973; Potts and Francoeur, 1990; Elias and Menon, 1991). Undoubtedly, the morphology of these lipids is a complex matter and has been proposed to be a two "phase" system with domains of lipids in the crystalline state surrounded by lipid interdomain border zones in the *liquid* crystalline state (Forslind, 1994).

The exceptionally low permeability of SC, however, is not necessarily exclusively due to the composition and morphology of the intercellular lipids. The unique architecture of the SC may result in highly tortuous extracellular pathways for the water molecules when diffusing through the SC (Michaels *et al*, 1975; Forslind *et al*, 1997). Furthermore, the complexity of the issue relates to other factors which include the permeabilities of the corneocytes, the lateral and vertical diffusion coefficients, and the SC/air, corneocyte/lipid matrix and the lipid/water (due to the heterogeneity of the lipid "phase") partition coefficients of water (Michaels *et al*, 1975; Heisig *et al*, 1996; Forslind *et al*, 1997; Johnson *et al*, 1997).

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Abbreviations: Azone, 1-dodecyl-azacycloheptan-2-one; CI, confidence interval; RH, relative humidity; SC, stratum corneum; TEWL, transepidermal water loss.

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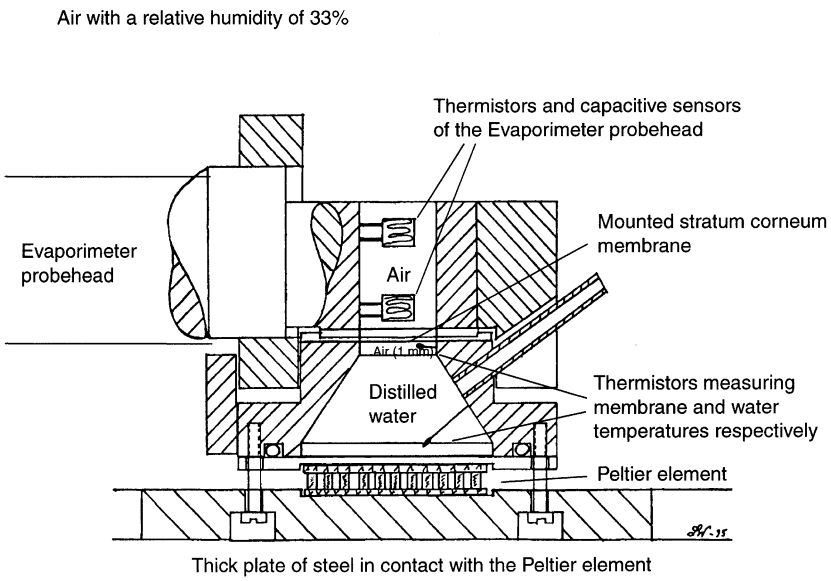

Figure 1. Evaporation unit, showing water chamber, mounted SC membrane, evaporimeter probehead.

The diffusion rate of a substance (F) through a membrane is given by Fick's first law of diffusion (Crank, 1975, pp. 49–53):

$$F = -D(\partial C/\partial x),$$

where D is the diffusion coefficient and $\partial C/\partial x$ is the concentration gradient over the membrane. In order to study the mass transfer (e.g., of water) through composite or structured media (e.g., lipid membranes) the experimentally determined rate of transfer can be expressed as a permeability coefficient, p, because a single diffusion coefficient cannot be used to describe the overall process (Crank *et al*, 1981). In general, the solute will have a finite solubility in the membrane, and consequently the concentrations at either side of the interface are governed by the equilibrium partition coefficient, σ (Crank *et al*, 1981). The effective (average) diffusion coefficient, D_{eff}, for a substance diffusing in a structured media may be estimated from the partition coefficient and the permeability coefficient according to (Crank *et al*, 1981):

$$p = D_{eff}\sigma.$$

Measurement of TEWL using the evaporimeter instrument is based on the fact that in the absence of forced convection, and neglecting the effect of thermal diffusion, the process of water exchange through a stationary water-permeable surface can be expressed in terms of the vapor-pressure gradient immediately adjacent to the surface (Eckert and Drake, 1959). The vapor-pressure gradient is approximately proportional to the difference between the vapor-pressure measured at two separate fixed points situated on a line perpendicular to the surface and in the zone of diffusion (Nilsson, 1977). The evaporimeter calculates the actual vapor pressure ($p = (RH)p_{sat}$) at each point of measurement, from the saturated vapor pressure (p_{sat}) (which is a function of temperature alone) obtained with a thermistor and the relative humidity (RH) obtained with a capacitive sensor (Nilsson, 1977).

The objective of our work was to construct and develop an *in vitro* system with high precision for fast quantitation of SC barrier function towards permeation of water. To evaluate the properties of our set-up we have used the system to quantitate the barrier perturbing effects on water permeation and water uptake induced by solvents (chloroform/methanol), divalent ions (Ca^{2+}), surfactants [SDS (sodium dodecyl sulfate)] and penetration enhancers [Azone (1-dodecyl-azacycloheptan-2-one), oleic acid].

MATERIALS AND METHODS

This study was carried out at a constant temperature of 21°C, justified by the fact that most of the data available from literature on lipid phase behavior is given at ambient temperature. Furthermore, the water diffusion rate at 21°C through isolated SC in contact with a water-filled tank exhibits values close to those of TEWL registered *in vivo* in normal skin (approximately 3–10 g per m^2 per h) (Norlén *et al*, 1999).

Materials The SC was isolated from breast skin from mammary reduction reconstructive surgery from a total of 36 subjects using a method previously described (Norlén *et al*, 1997). All water used was deionized using a Millipore equipment (Milli-Q-plus 185, Molsheim, France). $CaCl_2 \times 6H_2O$ (>99%, solubility 1 M in water at 20°C), SDS (>99%, M_w = 288.4 g per mol, solubility 0.1 M in water at 20°C), and oleic acid (>99%, M_w = 282.5 g per mol) were purchased from Sigma (St Louis, MO). Azone (>96%, M_w = 281.5 g per mol) was kindly provided by Whitby Research (Richmond, VA). All organic solvents were of high-performance liquid chromatography grade from J.T. Baker (Deventer, the Netherlands) and used without further purification.

Experimental set-up The mechanical parts including the evaporation unit (**Fig 1**), measurement box (**Fig 2**), extraction unit (**Fig 3**), sample holders (**Fig 4**), electronic control system, and computer software have been constructed and developed in our research department.

Discs with a diameter of 24.0 mm were punched out from the sheets of isolated SC and subsequently mounted in sample holders (**Fig 4**). The holders with mounted samples were then placed on a water-filled chamber, parallel to and 1 mm above the water surface (**Fig 1**). The evaporimeter probehead (EP 1A, Servomed, Mölndal, Sweden) was then locked to the water chamber, being in direct contact with the mounted SC membrane. The complete unit (water chamber, sample holder and evaporimeter probehead) was placed in a box with a constant relative humidity of 33.0% (controlled by a saturated solution of $CaCl_2 \times 6H_2O$ in distilled water) (**Fig 2**). The temperature of the mounted membrane was controlled by a computer system with a Peltier-element, constituting the floor of the water-filled chamber (**Fig 1**). The membrane temperature was set to 21°C in all runs. Water evaporation through the mounted pieces of isolated SC from breast skin was recorded once every 60 s during 10,000 s for each run, to ensure that the system had reached equilibrium conditions. The recorded values were sampled by the computer program and displayed graphically as a function of time (**Fig 5**). In addition the mean value of the last 2000 s (33.3 min) recording (8000–10,000 s, **Fig 5**) was calculated as a measure of water mass transfer through the membrane at steady state. Twenty runs were performed on a free water surface to gain reference data and to evaluate the precision of the method used.

All pieces were weighed before and after all runs to evaluate sample water uptake. After treatment with chloroform/methanol, 1 M solution of $CaCl_2$ in distilled water, 2 wt% SDS solution in water, Azone and oleic acid, respectively, the samples were left to dry and equilibrate overnight.

Water uptake equilibration test The SC samples were checked for steady-state *hydration* conditions at the time of water diffusion rate registration (8000–10,000 s, **Fig 5**). Samples from 30 subjects (20–59 y of age, median = 39) were divided into three groups and desiccated for 24 h before weighing. Subsequently, the samples of the first group (n = 10) were reweighed after 1 h mounting in the chamber, the samples of the

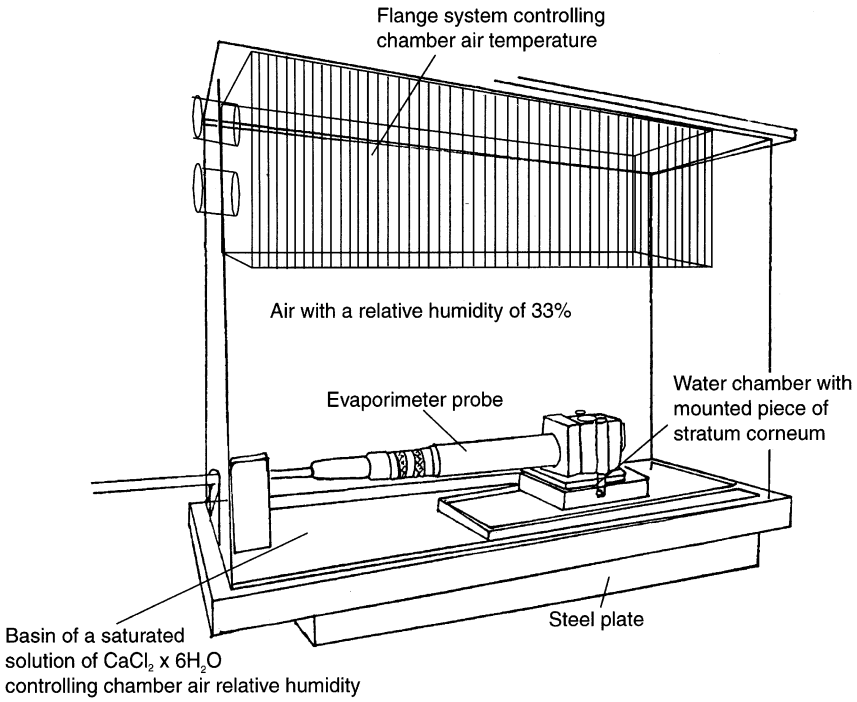

Figure 2. Measurement box, containing evaporation unit (Fig 1), basin of saturated solution of $CaCl_2 \times 6H_2O$, flange system.

second group (n = 10) after 2 h, and the samples of the third group (n = 10) after 3 h of mounting in the chamber.

Precision of TEWL measurements *in vivo* and *in vitro* As a means of comparing the precision of the *in vitro* water diffusion rate analysis system with TEWL measurements *in vivo* (following the guidelines of the standardization group of the European Society of Contact Dermatitis, Pinnagoda *et al*, 1990) one piece of SC was measured 10 times (once every 3 d during a period of 30 d) April–May 1998. Ten TEWL measurements *in vivo* (Evaporimeter EP1A, Mölndal, Sweden) on the mid forearm of one individual (L.N.) were performed in parallel with the *in vitro* measurements.

Main experiment

Lipid extraction Forty mounted pieces of SC, two pieces isolated from each of 20 subjects (2 × 20 = 40) (20–66 y of age, median = 42), were placed on the water-filled chamber. Subsequently, for each sample the water diffusion rate was recorded for 10,000 s (167 min) before and after lipid extraction. A total of 80 runs (2 × 2 × 20 = 80) were thus performed. The mounted samples were extracted, under continuous stirring, by immersion in three different combinations of chloroform/methanol (2:1 vol/vol, 1:2 vol/vol, 1:1 vol/vol) for 30 min each (3 × 30 min = 90 min). All pieces were weighed before and after lipid extraction and immediately before and after the water diffusion rate was recorded.

$CaCl_2$ and SDS Thirty pieces, three from each of 10 individuals (different from the individuals of the lipid extraction experiment) (19–64 y of age, median = 49), were divided into three groups of 10 pieces, one from each of the 10 individuals. The water diffusion rate through the pieces of the first group was recorded before and after immersion in distilled water for 30 min under continuous stirring. The water diffusion rate through the pieces of the second and third groups was recorded before and after immersion in a 1 M solution of $CaCl_2$ in distilled water and a solution of 2wt% SDS in water for 30 min, respectively. All pieces were weighed before and after immersion in the different immersion media and immediately before and after the water diffusion rate was recorded.

Penetration enhancers Twenty-four pieces, four from each of six individuals (different from the individuals of the lipid extraction experiment and the $CaCl_2$ and SDS experiment) (24–65 y of age, median = 44), were divided into two groups of 12 pieces (two pieces from each the six subjects in each group). The water diffusion rate through the pieces of the first group was recorded before and after application of one drop (using a Pasteur pipette) of Azone on the outer SC surface of the mounted samples. The Water diffusion rate through the pieces of the second group was recorded before and after application of one drop (using a Pasteur pipette) of oleic acid. All pieces were weighed before and after application of the different penetration enhancers and immediately before and after the water diffusion rate was recorded.

Statistics Means and regression coefficients were given with 95% confidence intervals (Snedecor and Cochran, 1980, p. 66). Residual mean squares were calculated to evaluate the instrumental variance (Snedecor and Cochran, 1980, p. 232). Analysis of variance with least significance difference *post hoc* comparisons, based on the studentized range, were used to compare the different treatments (Snedecor and Cochran, 1980, pp. 234–235). Regression analysis was used to test linear relationships (Snedecor and Cochran, 1980, Ch. 9). All tests of hypothesis were performed on the 5% significance level ($\alpha = 0.05$). A STATISTICA 5.1 software (Statsoft, Tulsa, OK) was employed for statistical calculations.

RESULTS

Evaporimeter system The water evaporation from a free water surface (water diffusion rate without interpositioned membrane structure), at 21°C and 33% relative humidity, was 41.0 ± 0.7 g per m^2 and h (95% CI of mean; n = 20) corresponding to a precision (random error) of 3.9%. The system reached an equilibrium level before 6000 s (100 min) (**Fig 5**) with no exceptions. This was expected as the maximum time lag (τ_{lag}) (Crank, 1975, p. 51) for water diffusion across the SC *in vitro* has been given as approximately 240–720 s (Scheuplein and Blank, 1971) and *in vivo* approximately 200 s (Kalia *et al*, 1996). The steady state is reached after approximately $3\tau_{lag}$ (Crank, 1975, p. 53).

The precision of the water diffusion rate measurements through mounted SC samples was 11% [TEWL being 1.9 ± 0.1 g per m^2 and h (95% CI of mean; n = 10)], the corresponding value for the TEWL measurements *in vivo* on human left forearm was 36% (TEWL being 8.7 ± 1.9 g per m^2 and h (95% CI of mean; n = 10), ambient relative humidity being 43%–56%, median = 47.5%, temperature being 20.5–21.5°C, median = 21.0°C).

Using regression analysis, no statistically significant linear trend in water uptake was seen between 1, 2, and 3 h of application in the chamber (95% CI of $\beta = 0.02 \pm 0.37$; n = 30). The mean relative water content to dry weight of the isolated SC after mounting in the chamber was 17.3 ± 2.3% (95% CI of mean; n = 30).

A condensed water film was present on the lower surface of all the pieces of SC during all runs.

Lipid extraction Water diffusion rate (n = 20), sample weight (n = 20) and water uptake (n = 20) before and after lipid extraction

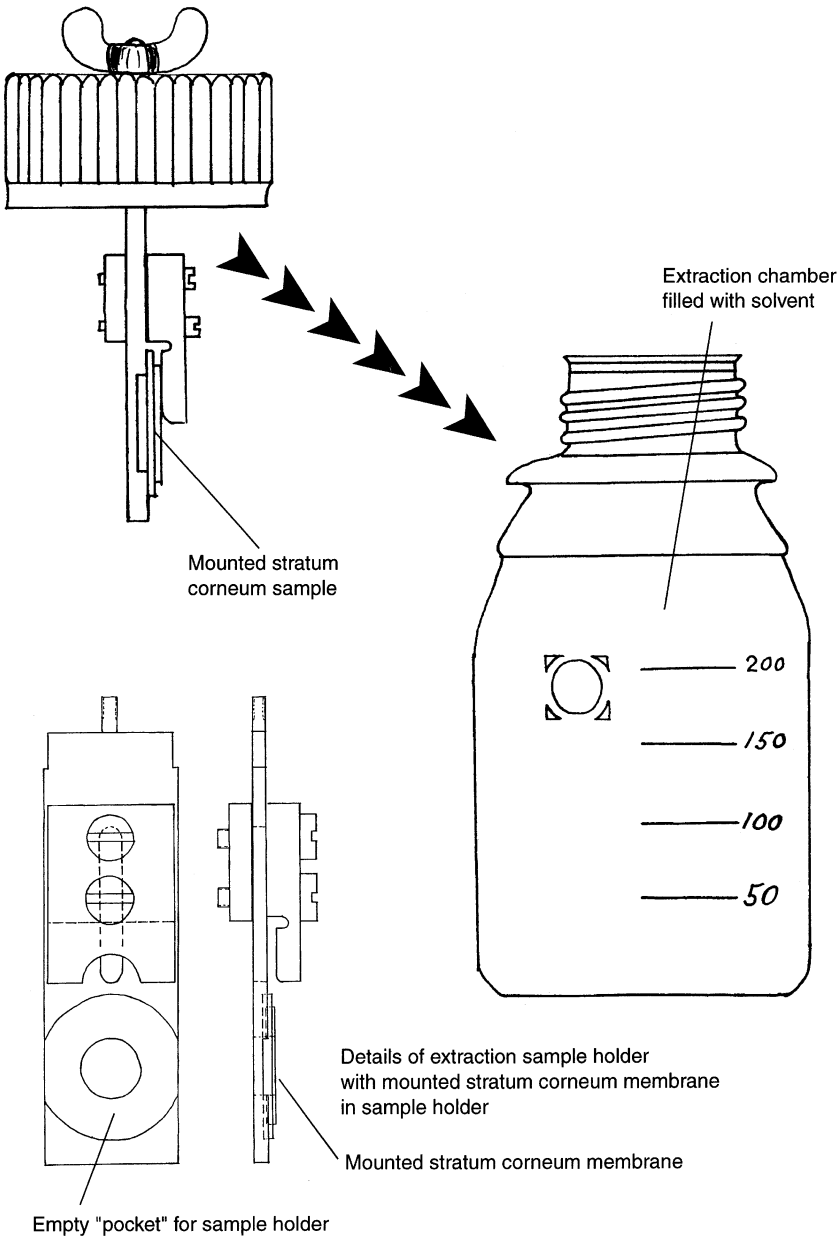

Figure 3. Extraction unit, showing solvent container, extraction sample holder, sample holder with mounted SC sample.

by three different combinations of chloroform/methanol is presented in **Table I**. A typical run of 10,000 s [167 recordings (min)] is presented in **Fig 5**.

The *difference* in water diffusion rate before and after extraction was 6.3 ± 1.3 g per m^2 and h (95% CI of mean; n = 20) and thus statistically significantly different from zero on the 5% significance level. The average relative *increase* in water diffusion rate after lipid extraction was approximately 250%, the water diffusion rate before and after lipid extraction being 2.5 ± 0.6 g per m^2 and h and 8.8 ± 1.5 g per m^2 and h (95% CI of mean; n = 20), respectively. This corresponds to an effective diffusion coefficient of 0.88×10^{-9} and 3.07×10^{-9} cm^2 per s^{-1}, respectively, before and after lipid extraction (assuming $\sigma = 0.063$ (Potts and Francoeur, 1991), $\Delta C = 1.0$ g per cm^3 (Pirot *et al*, 1998) and average skin thickness being 8 μm [calculated from the area of 452 mm^2, a SC tissue density of approximately 1 g per cm^3 (Anderson and Cassidy, 1973) and average sample weights, **Table I**)].

The amount of lipid extracted from desiccated SC by chloroform/methanol was 0.43 ± 0.15 mg (95% CI of mean; n = 20) corresponding to approximately 0.38 mg per cm^2.

The *difference* in water uptake (during water evaporation measurements for 10,000 s) before and after lipid extraction was – 0.07 ± 0.08 mg (95% CI of mean; n = 20) and thus not statistically significantly different from zero on the 5% significance level.

Using regression analysis, on the 5% significance level there was no statistically significant linear trend between sample weight (and thus average sample thickness) and water diffusion rate neither before nor after lipid extraction (95% CI of $\beta_{\text{intact sc}} = 0.001 \pm 0.69$; n = 20; 95% CI of $\beta_{\text{extracted sc}} = -0.29 \pm 0.66$; n = 20).

SDS and $CaCl_2$ Water diffusion rate, sample weight and water uptake before and after immersion in distilled water, 2wt% SDS in water and a 1 M solution of $CaCl_2$ in water for 30 min under continuous shaking (n = 10) is presented in **Table II**.

Using analysis of variance with *post hoc* least significant difference test based on the studentized range (Snedecor and Cochran, 1980, pp. 234–235), of differences between sample means (n = 10), there was a statistically significant difference in water diffusion rate between immersion in distilled water and immersion in a 1 M solution of $CaCl_2$ in water ($p < 0.05$, **Table III**). There was no statistically significant difference in water diffusion rate, however, between immersion in distilled water and 2wt% SDS in water ($p > 0.05$, **Table III**).

In our sample, the average relative *increase* in water diffusion rate

after immersion in a 1 M solution of $CaCl_2$ in water was approximately 130% and after immersion in 2wt% SDS in water approximately 50%.

There was a statistically significant difference in sample weight gain between immersion in distilled water and immersion in a 1 M solution of $CaCl_2$ in water ($p < 0.05$, **Table III**). There was no statistically significant difference in weight gain, however, between immersion in distilled water and 2wt% SDS in water ($p > 0.05$, **Table III**). In our sample, the average relative *increase* in weight after immersion in a 1 M solution of $CaCl_2$ in water was approximately 625% and after immersion in 2wt% SDS approximately 5%. Immersion in distilled water resulted in an average relative *decrease* in weight of approximately 5%.

There was no statistically significant difference in water uptake between immersion in distilled water and immersion in a 1 M solution of $CaCl_2$ in water ($p > 0.05$, **Table III**) and between immersion in distilled water and 2wt% SDS ($p > 0.05$, **Table III**). In our sample, the average relative *decrease* in water uptake after immersion in a saturated solution of $CaCl_2$ was approximately 80% and after immersion in 2wt% SDS approximately 10%. Immersion in distilled water resulted in an average relative *decrease* in water uptake of approximately 30%.

Penetration enhancers Water diffusion rate, sample weight, and water uptake before and after application of Azone and oleic acid, respectively, are presented in **Table IV**.

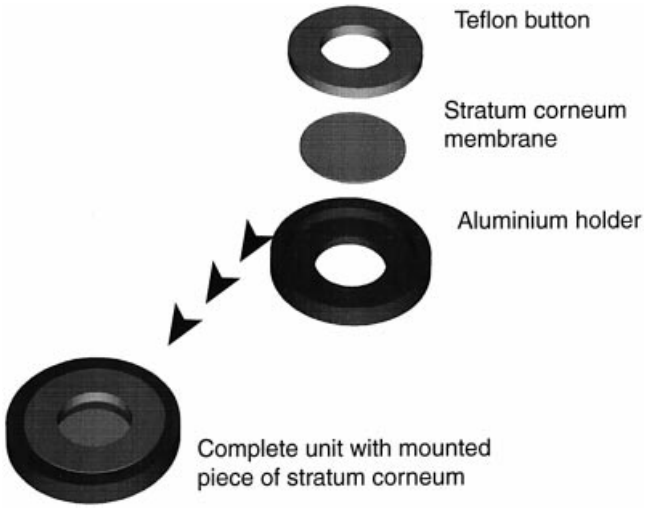

Figure 4. Sample holder composed of aluminum with a Teflon button firmly fixing the SC sample. Note that the vertical dimensions are exaggerated to promote visibility. The actual thickness of the complete unit is 1.0 mm.

Application of Azone caused a statistically significant decrease in water diffusion rate and water uptake (**Table IV**). Application of oleic acid had no statistically significant effect either on water diffusion rate nor on water uptake (**Table IV**).

Regression analysis showed that, on the 5% significance level there was no statistically significant linear trend between sample weight (and thus average sample thickness) and water diffusion rate neither before nor after application of Azone or oleic acid, respectively (95% CI of $\beta_{Azone} = 0.26 \pm 0.95$; n = 6; 95% CI of $\beta_{oleic\ acid} = -0.59 \pm 0.79$; n = 6).

DISCUSSION

For clinical use, no unbiased method is presently at hand for evaluation of the physical nature of the skin barrier towards water permeability. Our goal has been to develop a method to evaluate skin barrier function measured as water permeation, under highly controlled conditions. Body skin temperature, surrounding temperature and relative humidity as well as psychologic status and amount and activity of sweat glands, etc., are parameters of the *in vivo* situation that are difficult to control and standardize and liable to sudden changes. At the same time they exert the greatest influence on the evaporation of water from the skin surface (TEWL) (cf. Mathias *et al*, 1983). Our efforts have therefore turned towards the development of an *in vitro* system. In the controlled *in vitro* situation the evaporimeter has a random error (≈10%) of approximately one-third of that in the *in vivo* situation (≈35%) and hence it can detect small differences between test populations. In fact, for significant results an instrumental error of approximately 35% will necessitate either very big differences between the studied populations or very big samples. Consequently, the low instrumental error of our new *in vitro* method will not only make possible identification of barrier-related treats that are not possible to detect with the *in vivo* method, but it will also significantly reduce the sample sizes of investigations, with resulting time and economical gains.

In vivo, the term "barrier function" of the skin should be used with caution, as an increase in TEWL does not necessarily imply that the SC is altered (Léveque *et al*, 1993). Different processes that result in changed water concentration profiles over the SC (e.g., irritation induced by solvents, surfactants or by skin diseases such as atopic dermatitis) will automatically influence the obtained TEWL values. Given (i) the low precision of the *in vivo* method, and (ii) the subtle changes in barrier function of the SC measured *in vitro* (in comparison with *in vivo* data) after, e.g., exposure to anionic surfactants like SDS, it may be relevant to regard TEWL *in vivo* as an indicator of skin irritation rather than of barrier function *per se*. Measurements of the water diffusion rate through SC *in vitro* under controlled conditions, however, give accurate and precise information about the actual barrier function. The

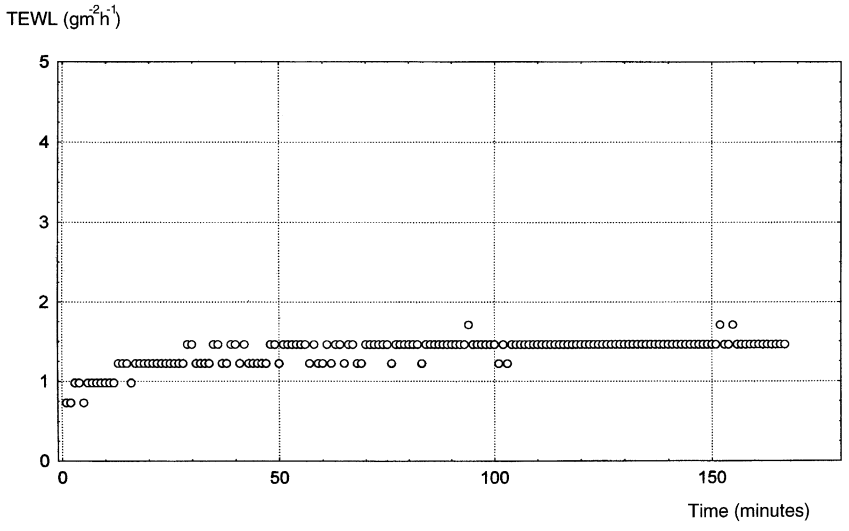

Figure 5. Mass transfer of water through an untreated piece of isolated SC as a function of time. The recording was performed at a temperature of 21°C, and a relative humidity of 33%.

Table I. Water diffusion rate, sample weight and water uptake before and after lipid extraction[a]

Sample	Water diffusion rate (g per m^2 and h)	Sample weight (mg)	Water uptake (mg)	CI for regression coefficients β between sample weight (independ.) and water diffusion rate (depend.)
Intact stratum corneum	2.5 ± 0.6	3.48 ± 0.83	0.26 ± 0.11	0.001 ± 0.69
Lipid extracted SC	8.8 ± 1.5	3.05 ± 0.93	0.19 ± 0.07	–0.29 ± 0.66
Difference between before and after extraction	6.3 ± 1.3	–0.43 ± 0.15	–0.07 ± 0.08	0.21 ± 0.68

[a]Water diffusion rate (n = 20), sample weight (n = 20), water uptake (n = 20) and linear correlation between sample weight and water diffusion rate (n = 20) before and after lipid extraction by three different combinations of chloroform/methanol (95% CI of mean and 95% CI of β, respectively). The regression coefficients are not significantly different from zero as the 95% CI of β includes zero.

Table II. Water diffusion rate, sample weight, and water uptake before and after immersion[a]

Sample	Water diffusion rate (g per m^2 and h)	Sample weight (mg)	Water uptake(mg)
Intact SC	4.7 ± 1.3	3.34 ± 1.17	0.33 ± 0.15
After immersion in distilled water	4.8 ± 2.0	3.21 ± 1.07	0.22 ± 0.17
Intact SC	3.9 ± 1.2	3.37 ± 1.16	0.45 ± 0.26
After immersion in 2wt% SDS	5.7 ± 1.8	3.56 ± 0.90	0.41 ± 0.30
Intact SC	3.7 ± 1.9	4.19 ± 0.79	0.62 ± 0.24
After immersion in a 1 M $CaCl_2$	8.5 ± 4.0	30.47 ± 6.65	0.11 ± 6.82

[a]Water diffusion rate, sample weight and water uptake before and after immersion in distilled water, 2wt% SDS in water and a 1 M solution of $CaCl_2$ in water for 30 min under continuous shaking (n = 10, 95% CI of mean).

Table III. Least significance difference *post hoc* comparisons of differences between sample means of water diffusion rate, sample weight, and water uptake before and after immersion[a]

	Distilled water	2wt% SDS in water
Water diffusion rate (g per m^2 and h)		
2wt% SDS in water	$p > 0.05$	
1 M $CaCl_2$ in water	$p < 0.05$	$p > 0.05$
Sample weight (mg)		
2wt% SDS in water	$p > 0.05$	
1 M $CaCl_2$ in water	$p < 0.05$	$p < 0.05$
Water uptake (mg)		
2wt% SDS in water	$p > 0.05$	
1 M $CaCl_2$ in water	$p > 0.05$	$p > 0.05$

[a]Least significance difference *post hoc* comparisons, based on the studentized range, of differences between sample means of water diffusion rate, sample weight, and water uptake before and after immersion in distilled water, 2wt% SDS in water and a 1 M solution of $CaCl_2$ in water.

main disadvantages of the *in vitro* method are that: (i) preparation of the SC samples is time-consuming, and (ii) skin samples of an appropriate size for measurements may not be readily available.

A question not yet fully answered is how extensive is the contribution of the intercellular lipid compartment to the overall SC barrier function? After extraction of the noncovalently bound lipids of the intercellular lipid compartment we recorded an *increase* in water diffusion rate of approximately 250% [2.5–8.8 g per m^2 and h (**Table I**)]. This actually corresponds to a *decrease* in total barrier capacity of 16% (the reference water flux from a free water surface being 41 g per m^2 and h for our set-up). The remaining 84% thus seems to be correlated to the protein envelope of the corneocytes (including covalently bound ceramides) and hence the three-dimensional architecture of the SC. The lipid extraction, however, may not have been uniform and complete (which in fact is unlikely after 90 min of extraction with chloroform/methanol). Clearly, our results indicate that the intercellular lipid compartment does not alone contribute to the SC barrier towards water permeation.

Comparing experimental results with data in the literature calls for a calculation of effective diffusion coefficients. In those cases where only permeability constants per unit length ($p = \sigma D_{eff}/l$) are given in the literature, we have for our comparisons performed transformations into approximate effective diffusion coefficients, taking $\sigma_{sc/w}$ to 0.063 and the membrane thickness to 10 μm (where nothing else has been stated). In this study, the measured average effective diffusion coefficients for normal and lipid depleted SC were $D_{eff\ normal\ SC} = 0.9 \times 10^{-9}$ cm^2 per s, $D_{eff\ extracted\ SC} = 3.1 \times 10^{-9}$ cm^2 per s, respectively (**Table V**). Corresponding *in vitro* data in the literature have given $D_{eff\ normal\ s.c}$ to 0.5×10^{-9} cm^2 per s (Scheuplein and Ross, 1970), 0.3–1.0 $\times 10^{-9}$ cm^2 per s (Blank *et al*, 1984) and, using a membrane thickness of 10 μm, 2.6×10^{-9} cm^2 per s (Potts and Francoeur, 1991), respectively (**Table V**). *In vivo* experiments yielded $D_{eff\ normal\ s.c}$ 4.4 ± 2.0 $\times 10^{-9}$ cm^2 per s (Kalia *et al*, 1996; Pirot *et al*, 1998). Corresponding values for lipid *extracted* SC *in vitro* has been reported to $D_{eff\ extracted\ SC} = 3.2 \times 10^{-9}$ cm^2 per s (Abrams *et al*, 1993) and 50×10^{-9} cm^2 per s (Scheuplein and Ross, 1970) (**Table V**). This latter value is still approximately three orders of magnitude lower than the self-diffusion of liquid water in water (2.31×10^{-5} cm^2 per s at 25°C, Hertz, 1973). The remarkably high effective diffusion coefficient of extracted SC reported by Scheuplein and Ross as compared with our results (**Table V**) may be explained by differences between the techniques employed.

SDS in water has been reported: (i) to increase the permeability of water through human epidermis; (ii) to reduce the water-holding capacity; and (iii) to cause structural damage (partly reversible) to both the protein and lipid compartments of the SC (Blank and Shappirio, 1955; Bettley and Donoghue, 1960; Scheuplein and Ross, 1970; Imokawa *et al*, 1989; Wilhelm *et al*, 1993). Our data seem to challenge the two first statements (**Table III**). The differences in earlier reported *in vitro* results to our findings, however, may partly be due to different techniques used to determine SC water permeabilities. Also, the reported *in vivo* results can partly be explained by an hyperhydration of the SC consecutive to an irritant reaction induced by the surfactant (Léveque *et al*, 1993; Wilhelm *et al*, 1993), and thus does not necessarily contradict our data. It has been demonstrated that the increased skin surface water evaporation *in vivo* is *not* related to lipid extraction of the SC by SDS (Léveque *et al*, 1993; Fartasch *et al*, 1998). Also, a disorganizing effect imposed by the surfactant molecules on the intercellular lipid bilayers seems unlikely as the upper portions of SC have been shown to display intact intercellular lipid layers after exposure to SDS *in vivo* (Fartasch *et al*, 1998). One possible effect may be that the surfactant molecules mainly interact in the interzone between the lipid and protein compartment or directly on the SC proteins (i.e., intracellularly or at the corneocyte envelopes) (Scheuplein and Ross, 1970; Léveque *et al*, 1993). This tentative

Table IV. Water diffusion rate, sample weight and water uptake before and after application of Azone and oleic acid[a]

Sample	Water diffusion rate (g per m^2 and h)	Sample weight (mg)	Water uptake (mg)
Intact SC	3.2 ± 0.9	2.27 ± 0.15	0.20 ± 0.07
After application of Azone	2.2 ± 0.9	21.91 ± 5.04	–2.63 ± 2.19
Difference between before and after Azone – application	–1.0 ± 0.9	19.64 ± 5.05	–2.82 ± 2.19
Intact SC	1.1 ± 2.53	3.80 ± 0.81	0.41 ± 0.27
After application of oleic acid	–0.3 ± 0.4	17.04 ± 1.31	–0.60 ± 1.87
Difference between before and after oleic acid-application	–1.4 ± 2.9	13.24 ± 1.30	–1.01 ± 1.77

[a]n = 6, 95% CI of mean.

Table V. Comparison of our effective diffusion coefficients with recalculated effective diffusion coefficients in the literature

$D_{eff\ normal\ s.c}$ (cm^2 per s) × 10^9	$D_{eff\ extracted\ s.c}$ (cm^2 per s) × 10^9	Experimental set-up	Reference
0.9	3.1	Evaporimeter, *in vitro*; human SC; at 21°C, RH 33%; 20 subjects	This study
0.5	50	Diaphragm diffusion cell technique, tritium (HTO); *in vitro*; human epidermis; at 25°C, direct water contact with membrane; number of subjects not given	Scheuplein and Ross, 1970
0.3–1.0		Diffusion cell, tritium (HTO); *in vitro*; human SC; at 31°C, RH 46-93%; 3 subjects	Blank *et al*, 1984
2.6 (l taken to 10 μm)		Diffusion cell, tritium (HTO); *in vitro*; porcine SC; at 22°C, RH 75%; number of subjects not given	Potts and Francoeur, 1991
	3.2	Evaporimeter, *in vitro*; human full skin; at 18–24°C, RH 25-45%; direct water (saline 0.9% wt/vol, 37°C) contact with membrane; 3 subjects	Abrams *et al*, 1993
4.4 ± 2.0 (mean ± SD)		Evaporimeter, *in vivo*; ambient temperature and RH%; 3 and 13 subjects respectively	Kalia *et al*, 1996; Pirot *et al*, 1998 (*in vivo*)

notion is supported by the fact that most of the lipids in the intercellular compartment of the SC are in a crystalline state at ambient temperature and will have little tendency to interact with the surfactant molecules (cf. Engblom *et al*, 1995). Consequently, the bulk of the lipid lamellar structures will remain unperturbed. This notion supports our findings that both barriers function towards water diffusion and water-holding capacities of human SC are surprisingly unaffected even after prolonged exposure to surfactants (e.g., in this study completely submerged for 30 min in 2wt% SDS in water under continuous stirring). The small effect on sample weight of immersion in 2wt% SDS in water (**Table II**) may be explained by a concomitant extraction of protein material.

The immersion in 1 M $CaCl_2$ in water did statistically significantly increase the water diffusion rate. The increase in weight, however, was conspicuous and an increased water content of the SC, with a resulting increase in the effective water diffusion coefficient (Blank *et al*, 1984), may explain the registered increase in water evaporation (despite the thickness increase associated with swelling, Norlén *et al*, 1997). On the other hand, charged surfaces, e.g., lipid bilayers with ionized free fatty acids, can be efficiently neutralized through screening effects and ion exchange by divalent ions (e.g., Ca^{2+}) (cf. Israelachvili, 1992). Thus, divalent ions may exert an influence on the morphology of the intercellular lipid lamellae of the SC with possible deteriorating effects on barrier function.

Penetration enhancers like Azone and oleic acid have been shown to increase the penetration of both polar and nonpolar drugs through the skin (Barry, 1987; Wiechers, 1992; Schaefer and Redelmeier, 1996, pp. 168–172). Both Azone and oleic acid are immiscible with water. Furthermore, Azone has recently been found to favor liquid crystals of reversed morphology (Engblom and Engström, 1993), similar to the effect of oleic acid. A particularly interesting observation, which to some extent may explain their dual effect on the skin barrier, is their ability to induce a transformation from a lamellar liquid crystal into coexisting reversed micelles (i.e., an oil) and a lamellar phase of significantly higher water content (Engblom *et al*, 1995). In systems containing lipids in both crystalline and liquid crystalline state (cf. Forslind, 1994) they tend to interact predominately with the liquid crystals.

In addition, to exert an influence on skin barrier lipid morphology, application of excessive amounts of Azone or oleic acid may be expected to produce an occlusive layer on the skin surface. Our data show an occlusive effect of pure Azone, but not of oleic acid, when applied in excess amounts to isolated SC (**Table IV**). Further studies are needed to explain this discrepancy.

CONCLUSIONS

It is important to have reliable methods for evaluation of skin barrier function if questions such as barrier perturbing effects of different agents and occlusive effects of different formulations are to be elucidated. We have developed a new evaporimeter-based *in vitro* system for rapid measurements of SC barrier function with more than a 3-fold higher precision (lower random error) than measurements of TEWL *in vivo*. This experimental system provides a sensitive method for determination of barrier perturbation measured as TEWL.

Removal of the lipid intercellular matrix results in approximately a 3-fold increase in the water diffusion rate of the isolated SC, not a 100-fold as has previously been suggested. Our results indicate that the intercellular lipid compartment does not alone contribute to the SC barrier towards water permeation but that the protein envelope of the corneocytes (with covalently attached ceramides) may also be involved.

Exposure to anionic surfactants, as exemplified by submersion in 2wt% SDS in water for 30 min under continuous stirring, does neither alter the water diffusion rate nor the water-holding capacity of SC. This does not exclude the possibility that the permeability of other substances is altered.

Exposure to 1 M $CaCl_2$ in water yields an increased water diffusion rate through SC. One tentative explanation may be that divalent ions exert an influence on the morphology of the intercellular lipid lamellae of the SC with possible deteriorating effects on barrier function.

When applied to the SC in excess concentrations, the penetration enhancer Azone has occlusive effects on water diffusion through the SC, not penetration-enhancing effects.

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