

A new *in vitro* method for transepidermal water loss: A possible method for moisturizer evaluation

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Synopsis

Transepidermal water loss (TEWL) through the ventral skin of the hamster ear was determined using a modified flow-through diffusion apparatus. Tritium-labeled water was allowed to permeate through the dermal/epidermal layers. Water vapor was collected in a closed system by adsorption onto solid anhydrous calcium chloride in a separately attached receiver. The desiccant was removed, dissolved in water, and the radioactivity determined by liquid scintillation counting. The rate of TEWL was determined for various durations of exposure to the desiccated environment, in the temperature range between 5 and 40°C. TEWL rate reached a maximum one hour after exposure, and then decreased to a steady state with time. TEWL increased exponentially with increasing temperature in accordance with the Arrhenius relationship. An activation energy value of 13 Kcal/mole was obtained. At incubator temperature of 22°C, the rate of TEWL was found to be $152 \pm 14 \mu\text{g}/\text{cm}^2/\text{hr}$, which agrees with previously reported values obtained by *in vivo* methods of testing. The present technique is proposed as a rapid *in vitro* method for measuring TEWL and as a possible pretest for assessing efficacy of potential skin-moisturizing agents. Four agents were studied for their effect on water permeability. The agents tested were mineral oil, castor oil, sesame oil, and 25% glycerin in water. Skin membranes treated with occlusive agents such as mineral oil, castor oil, and sesame oil showed a marked decrease in the TEWL rate, while those membranes treated with the humectant, 25% glycerin in water, showed a marked increase in the TEWL rate. Similar results using these same agents have been reported previously.

INTRODUCTION

Blank showed that proper hydration of the stratum corneum is a primary factor in maintaining skin softness and flexibility (1,2). Other constituents of skin, such as proteins and lipids, play a minor role by influencing the water-binding capacity of the stratum corneum. Appearance and barrier function of the skin is affected, if it is not optimally hydrated (3). This effect is exhibited in low relative humidity environments, where the skin becomes dry, flaky, and/or chapped. When skin hydration increases at higher relative humidity, the water content and the permeability of skin increases (1,2,4–10). It is considered supple, flexible, pliable, and less likely to chap, fissure, and crack (1,2,11–14).

Some cosmetic chemists regard moisturization as any means to increase water content and maintain this level over a period of time (15). “A moisturizer,” as defined by Kligman (16) “is a topically applied substance or product that overcomes the signs and

symptoms of dry skin." In simple terms, the alleviation of the dry skin state after "moisturizers" are applied externally may be partly due to the increased hydration or water content of the skin (1). One of the methods used to assess the hydrated state of the skin is to measure the percent decrease of transepidermal water loss (TEWL) on skin treated with a moisturizer versus untreated skin. TEWL is a passive diffusional process where water vapor diffuses from highly hydrated dermal layers via the stratum corneum to the exterior surface where it evaporates freely.

Leveque *et al.* (17) recently made *in vivo* TEWL measurements (Servomed® evaporimeter technique of Nilson) of dry skin patients and compared this method to the regression method of Kligman (16). A statistically significant trend was observed with TEWL and dry skin, although the two did not correlate closely. It is noted that an increase in TEWL (from normal levels) during a dry skin state suggests a disturbance in the horny layer structure. However, during this state there's also a tendency of dry skin to form a thicker horny layer, counteracting this effect (17). This method of measuring moisturizing efficacy via TEWL rate decrease has its limitations and is a subject of controversy.

Other methods that have been used to hydrate the skin include (18): 1. applying external water directly to the stratum corneum, and 2. increasing the rate of diffusion of water from the lower epidermal layers through the stratum corneum.

According to Wu (19), transepidermal water loss is described by the term called flux and is expressed by Fick's second law:

$$J = D(C) \, dC/dX$$

where J equals flux, in $\mu\text{g}/\text{cm}^2/\text{hr}$, C equals water concentration in the stratum corneum, $\mu\text{g}/\text{cm}^3$, X equals the thickness of skin membrane, cm, and D(C) equals diffusivity of water in the stratum corneum, cm^2/hr . D(C) is a constant developed by Wu (19) to describe the diffusivity of water and its relation to the barrier properties of the membrane utilized.

FACTORS AFFECTING TEWL

The environmental temperature, relative humidity, and skin source must be controlled during experimentation in order to determine TEWL. This requirement is evident by the large variation in TEWL values, reported previously: 100–1500 $\mu\text{g}/\text{cm}^2/\text{hr}$ (1,4,6,9,20–26).

In vivo studies by Grice *et al.* (20,21) and *in vitro* experiments by Matias (22) have shown that TEWL will increase exponentially with increasing temperature. Reports of the effect of environmental relative humidity on TEWL, however, are in conflict (23–26). Using different experimental methods, Goodman and Wolf (23), Spruit and Malten (24), and Bettley and Grice (25) showed that the water loss through skin *decreases* as relative humidity increases. More recent controlled *in vivo* studies by Grice (26) revealed a maximum TEWL rate between 50% and 75% relative humidity (RH).

In vitro measurements with fetal hog periderm by Wu (19) have confirmed Grice's findings (26). The integrity and source of skin used in *in vitro* studies are also critical factors. It has been reported that diseased and damaged skin have increased TEWL (27–28). Dupuis (29) reported a TEWL value from forehead skin almost twice that from skin at other anatomic sites.

Application of topical vehicles can either increase or decrease the hydration of the stratum corneum depending on how the vehicle alters water activity in the barrier. Oily, occlusive materials, such as petrolatum, mineral oil, lanolin, and isopropyl myristate, significantly decrease the rate of water loss from the skin. These agents can be considered moisturizing agents since they are able to increase skin hydration. Humectants, agents that act to bind water to skin, such as glycerin and propylene glycol, can increase skin moisture by interacting with the atmospheric moisture in conditions of moderate to high humidity. When the relative humidity of air decreases, humectants on the skin, however, will extract moisture from the deeper layers. Rieger and Deem have shown that humectants increase the *in vitro* TEWL at conditions of low humidity (30). Humectants alone, therefore, only function as a moisturizer in proper atmospheric conditions. It is conceivable that the moisturizing emulsions can still decrease TEWL, at various humidities, even with a humectant present in the formula. Decreasing TEWL presumably increases the pool of water available to hydrate the skin. It is possible to determine the influence of TEWL on moisturizer effectiveness by comparing a TEWL value on untreated skin to that value obtained from moisturizer- or occlusive agent-treated skin (30).

In vitro methods for TEWL measurement can be utilized to pre-evaluate the potential efficacy of moisturizers in human skin. Present *in vitro* methods (1,2,4,7,8,19,30–33) are time-consuming to use for this purpose. Another limitation is that water loss from the skin can conflict with water loss from the moisturizer. The objective of the present research was to develop a simple and accurate method that permits rapid evaluation of TEWL of potential moisturizers by a quantitative measurement of moisture loss through an animal skin membrane. This method uses a tritiated-water tracer technique which can eliminate erroneous TEWL values that may result from the water evaporation from a moisturizer.

EXPERIMENTAL PROCEDURE

APPARATUS DESIGN

A modification of a flow-through diffusion cell originally designed by Bronaugh (34) was utilized. The membrane was full-thickness, cartilage-stripped skin taken from the ventral ear of the male Syrian golden hamster (Harlan-Sprague Dawley, Indianapolis, IN) as described by Matias (35). Tritiated water (HTO) permeated through the membrane into the receptor compartment of the diffusion cell. Flux (TEWL) was determined, via scintillation counting, by the measurement of HTO that adsorbed on anhydrous calcium chloride in the receptor cell. A TEWL rate-vs-time study was conducted to determine the time to steady state or equilibrium conditions at 32°C. The prepared cells were then exposed to temperature increments within the range between 5 and 37°C, and then TEWL measured at steady state. A plot of the logarithm of TEWL vs the reciprocal of absolute temperature was used to determine the energy of activation (by slope analysis) in accordance with the Arrhenius relationship.

An evaluation of TEWL was conducted for skin treated with three occlusive agents: mineral oil, castor oil, and sesame oil, and skin treated with a humectant (25% glycerin

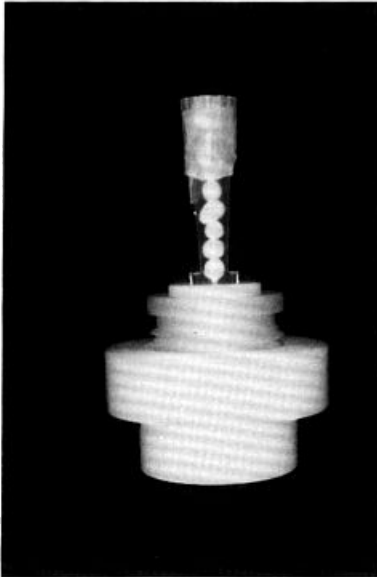
in water) by adding the 10 μL of the agent onto the exposed skin area (0.32 cm^2) and conducting a TEWL rate-vs-time study. A skin-conditioning study was also done. Hamster ear skin was treated *in vivo* with daily applications (10 $\mu\text{L}/\text{ear}$) of mineral oil for two weeks. Animals were then sacrificed and TEWL determined for the previously treated and untreated contralateral ear skins.

APPARATUS PREPARATION

Syrian golden hamsters were sacrificed by means of an intraperitoneal injection of sodium pentobarbital (65 mg per animal). The ears were removed by incision at the base with the aid of surgical scissors. The dorsal ear skin was gently pulled away from the supporting cartilage, starting at the base and extending distally. The cartilage was gently scraped off with a scalpel from the ventral side. A 10-mm diameter dermal punch was taken from the center of the stripped skin sample. A photograph of the teflon cell used for *in vitro* TEWL studies is shown in Figure 1. The cell was equilibrated to constant temperature at 32°C using heated water pumped through an aluminum holding block from a 35°C water bath or from a temperature-controlled incubator. The 10-mm punch biopsy of the skin was placed ventral or hairy side up on the lip or edge of the donor compartment and the top holder was screwed tightly into place. The inside section of the top was free to rotate so that the top could be secured without twisting the skin. The exposed area of the skin was approximately 0.32 cm^2 .

With the membrane tightly fitted in place, one side of the side-arm opening was closed with a petrolatum plug. The donor compartment of the experimental cell (section underneath skin) was filled from the opposite side with approximately 150 μL of 100 $\mu\text{Ci}/\text{ml}$ solution ($2.2 \times 10^8\text{ dpm}/\text{ml}$) containing tritiated water (HTO). This side was also closed with a petrolatum plug. A yellow Eppendorf pipet tip with a 5.0-mm section cut off from the bottom and, subsequently, attached into a 10-mm length section of $\frac{1}{4}$ " O.D., $\frac{1}{8}$ " I.D., $\frac{1}{16}$ " wall Tygon tubing served as the receptor compartment. The Tygon tubing end was attached to the screwed top portion of the diffusion cell (see Figure 1). The receptor compartment was filled with approximately 0.24 g of granular, anhydrous calcium chloride, and the top covered with Parafilm. The cell was returned to the heated aluminum holding block or the incubator, and the desiccant was removed or exchanged according to the time specified. The desiccant was placed in a 20-mL scintillation vial and dissolved in 2 ml of purified water. A 500- μL aliquot sample was then placed into a new vial, dissolved in 10 mL scintillation cocktail (Aquasol-2, New England Nuclear, Boston MA), and counted for tritium content for one minute in a Model SL400 Inter technique scintillation counter.

A known volume of HTO was dissolved in water and the counts per minute (cpm) determined by scintillation counting. This was designated as the reference standard. A background count was determined for a volume of water and subsequently subtracted from the count for the reference standard. Both the reference and the blank standard had the same concentration of dissolved calcium chloride as the sample. This was to eliminate erroneous values due to quenching. The cpm was determined for the known volume of HTO and divided by density to determine the cpm/weight. An HTO count value was determined for sample and divided by the reference value, the time, and membrane area to determine the TEWL in $\mu\text{g}/\text{cm}^2/\text{hr}$. Six replicates were prepared.



A. Receptor compartment

B. Donor compartment

C. Parafilm seal

D. Receptor compartment; filled with calcium chloride.

E. "Screw-in" attachment; functions to secure skin and hold receptor cell.

F. Skin membrane; placed upon small well of water which functions as donor compartment.

Figure 1. *In vitro* TEWL apparatus, representing a modification of Bronaugh's flow-through diffusion cell.

RESULTS AND DISCUSSION

METHODOLOGY

Calcium chloride was chosen as it is the most convenient desiccant available for study. Five individual measurements were made of the amount of anhydrous calcium chloride placed in the receptor compartment of the cell. The average weight recorded was 0.24 ± 0.016 g.

Cartilage-stripped hamster ear membrane is a new source for obtaining a fresh sample of full-thickness skin membrane. Percutaneous absorption theory states that the flux is inversely proportional to skin thickness (10). In actuality, we are referring to the thickness of the stratum corneum, as this is the rate-determining skin layer. Measured skin thickness was 0.181 ± 0.003 mm. Since little or no variation in skin thickness was observed, it was assumed to be a constant when measuring TEWL.

TIME STUDY

Two blank and six experimental cells were prepared and placed into the heated aluminum holding block at various temperatures. The receptor compartment containing the desiccant was exchanged for a fresh sample at the following time intervals: 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, and 20 hours. The TEWL flux was determined by calculating the cumulative counts at the particular time interval.

A plot of the rate of TEWL-vs-time is shown in Figure 2. It was observed that at 0.5 hour and one hour, the rate increased rapidly from $340 \mu\text{g}/\text{cm}^2/\text{hr}$ to $460 \mu\text{g}/\text{cm}^2/\text{hr}$, and then the TEWL decreased after two hours. Equilibrium was reached between the third and sixth hour. The apparatus operated in the presence of a dry atmosphere (less than 25 percent relative humidity), determined by a cobalt chloride paper technique

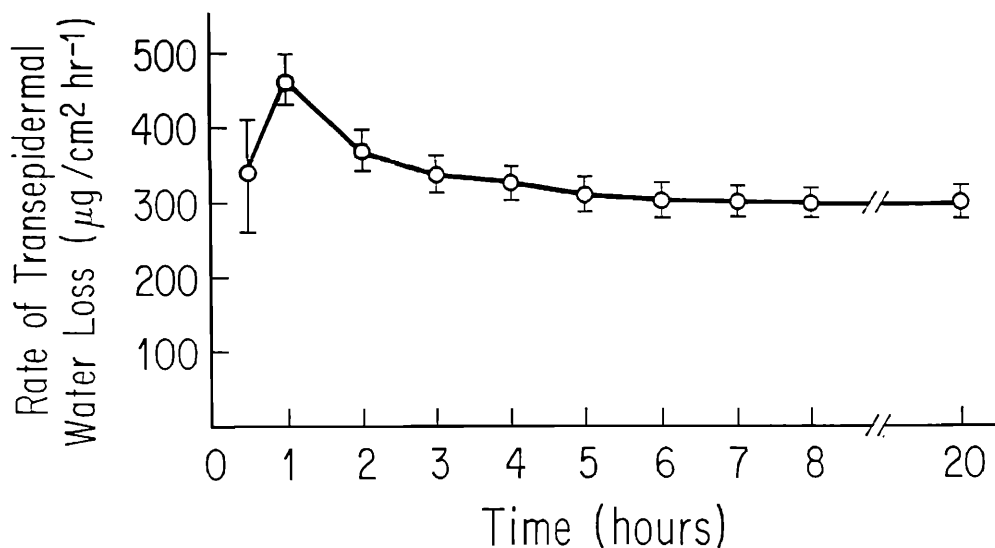


Figure 2. TEWL rate vs time. Bars refer to standard error of measurement.

adapted from Solomon (36). Since the apparatus operates in the presence of a dry atmosphere, this transit time period results in an initial evaporation of water from the membrane. Initially, the membrane is fully hydrated, swollen, and is more permeable. This provides an "increased push" effect to pass water through the membrane towards the anhydrous desiccant. This transit time period is designated the "burst" effect. At steady state, the amount of water adsorbed onto the desiccant is in equilibrium with the amount above the membrane. Also, the membrane is approaching a drier state and is less permeable.

Equilibrium TEWL rate after six hours was approximately $300 \mu\text{g}/\text{cm}^2/\text{hr}$ at 32°C . Table I summarizes previously reported TEWL values based upon the various *in vitro* techniques currently employed for measurement.

Blank (1,2) utilized full-thickness skin membrane from the human abdomen in his studies and reported TEWL values of between 130 and $270 \mu\text{g}/\text{cm}^2/\text{hr}$ (23°C , 23% RH). Our value of $152 \mu\text{g}/\text{cm}^2/\text{hr}$ (22°C , 20–25% RH) for the hamster skin is in excellent agreement with Blank's value for human skin.

TEMPERATURE STUDY

A plot of TEWL rate-vs-temperature is shown in Figure 3. An exponential increase in TEWL was observed, which implies that water vapor permeates by a diffusional process. The TEWL rate between 5 and 12°C , however, showed little change.

Table I
Summary of Transepidermal Water Loss (TEWL) Rate Data for Various *In Vitro* Skin Models

Investigator	Year	Reference	Membrane used	Method	TEWL rate ($\mu\text{g}/\text{cm}^2/\text{hr}$)	Temperature $^\circ\text{C}$	Relative humidity in %
Blank	1952	(1,2)	Human, full-thickness skin from abdomen	Gravimetric	100–200	23	40–50
					130–270	23	20–30
					400–800	35	57
Mali	1956	(8)	Human, epidermis from trunk	Gravimetric, desiccator	500–600	21	0
Mali	1956	(8)	Human, epidermis from sole	Gravimetric, desiccator	3000	21	0
Reiger, Deem	1974	(30)	Human, stratum corneum from abdomen	Gravimetric	250–300	21	0–20
Wu	1983	(19)	Fetal hog periderm	Saturated salt solutions	251	21	50
					130	21	40
					85	21	30
Blank <i>et al.</i>	1984	(33)	Human, stratum corneum	Saturated salt solutions and tritiated water	600–1200	30	0–80
This work	1987		Hamster ear, full-thickness skin	Tritiated water	250–300	32	20–25
					152	22	20–25

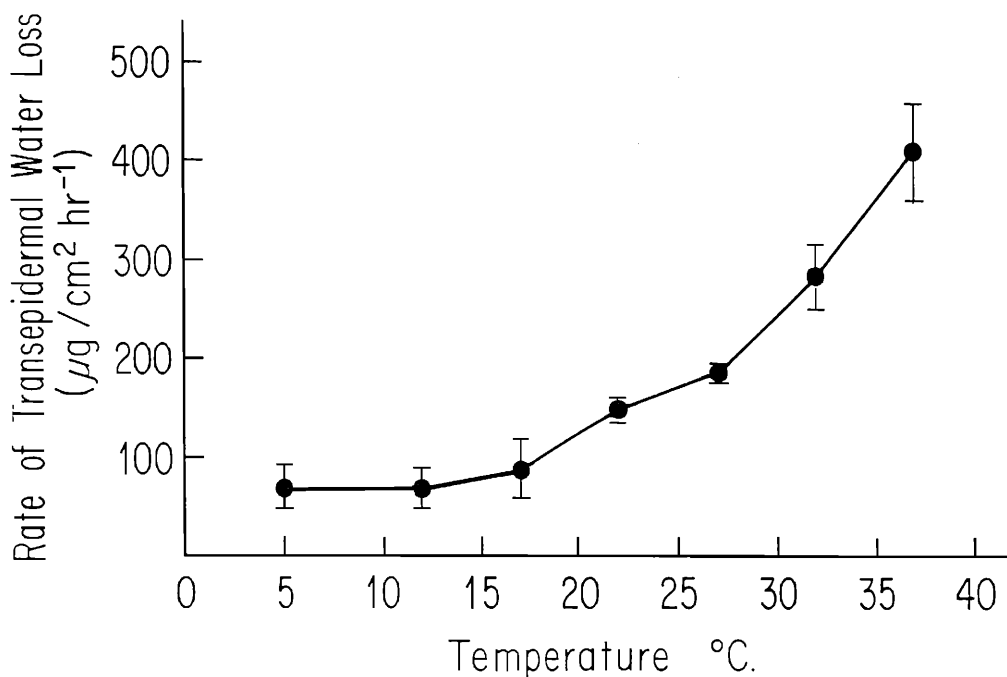


Figure 3. TEWL rate vs temperature at steady-state conditions (after 3 hours). Bars refer to standard error of measurement.

If the reciprocal temperatures in degrees Kelvin are plotted vs the natural logarithm of the TEWL rate in the temperature ranges between 12 and 37°C, linearity is observed (Figure 4). It is difficult to explain the lack of linearity at 5°C. The water permeation may have been disrupted at this near-freezing temperature. The energy of activation was calculated by slope analysis. Our calculated value of 13.1 Kcal/mole compares favorably with an activation energy value of 15 Kcal/mole previously reported by Scheuplein for human skin (9).

MOISTURIZER STUDY

Four experimental cells and two blank cells were prepared for each moisturizing agent tested. Ten μL of each agent was applied to the top of the membrane surface, prior to attachment of the receptor cell filled with desiccant and placement of the unit into the 32°C heating block. Plots of TEWL-vs-time values for membranes treated with various potential moisturizers are shown in Figures 5 and 6. The figures show individual TEWL values vs time over a 24-hour time period for the untreated sample and skin treated with agents. The untreated sample shows a result similar to that reported in Figure 2. Transepidermal water loss reaches steady-state conditions after three hours, except for that sample treated with 25% glycerin.

When compared to the untreated sample, castor oil is the only agent that exhibited a "burst" effect. Skin treated with sesame oil, mineral oil, and 25% glycerin in water all appear to inhibit this effect. Sato and Nagai (37) compared the effects of different

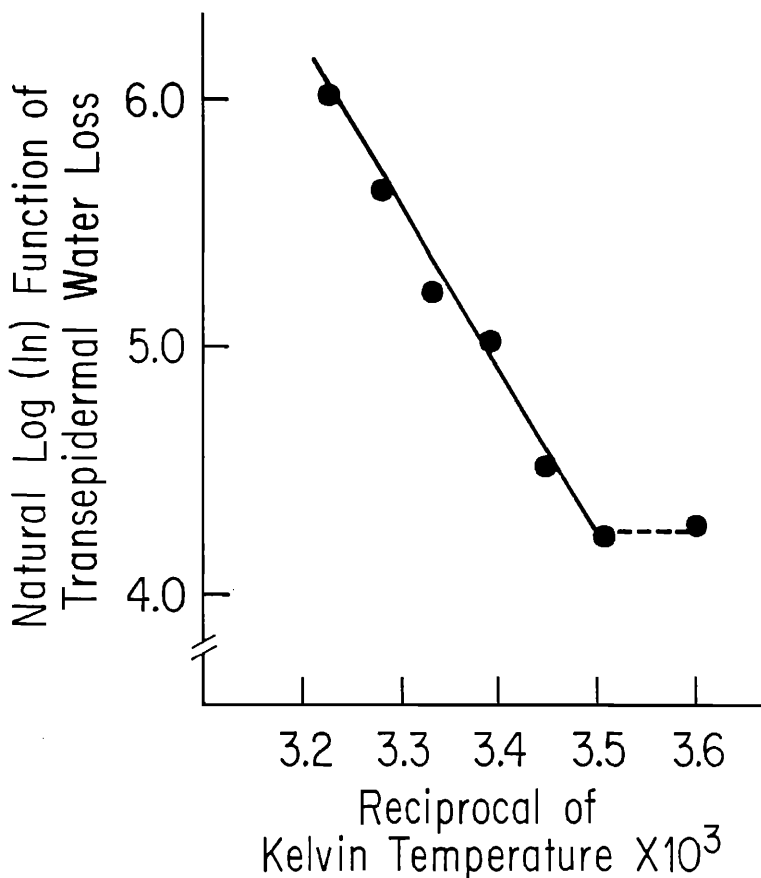


Figure 4. Natural logarithm of TEWL vs reciprocal of absolute temperature $\times 1000$.

emollient materials on TEWL using a *silastic* membrane. There was a good correlation between the TEWL rate for this *in vitro* model and the TEWL rate for an *in vivo* human skin model. They found that the decrease of TEWL rate that resulted from emollient application was inversely proportional to the polarity of the material applied. In other words, the more polar the material applied to the membrane, the greater the TEWL rate. Mineral oil is the least polar substance that we tested and it inhibited TEWL the most. At steady state, the effect of castor-oil-treated skin on TEWL (Figure 6) was similar to that of sesame-oil-treated skin (Figure 5).

Glycerin, which acts to bind water to the skin, produced an interesting effect. During the first two hours glycerin acted as an occlusive agent, since TEWL values were close to that of mineral-oil-treated skin. After four hours, however, the glycerin-tissue composite was observed to become saturated with water. At the six-hour time point, TEWL rates were almost double those of untreated skin. This is comparable to the results obtained by Reiger and Deem (30) who found an increase in the TEWL rate upon application of 25% glycerin in water. Reiger and Deem showed that humectants, in general, increase TEWL rate at low relative humidities.

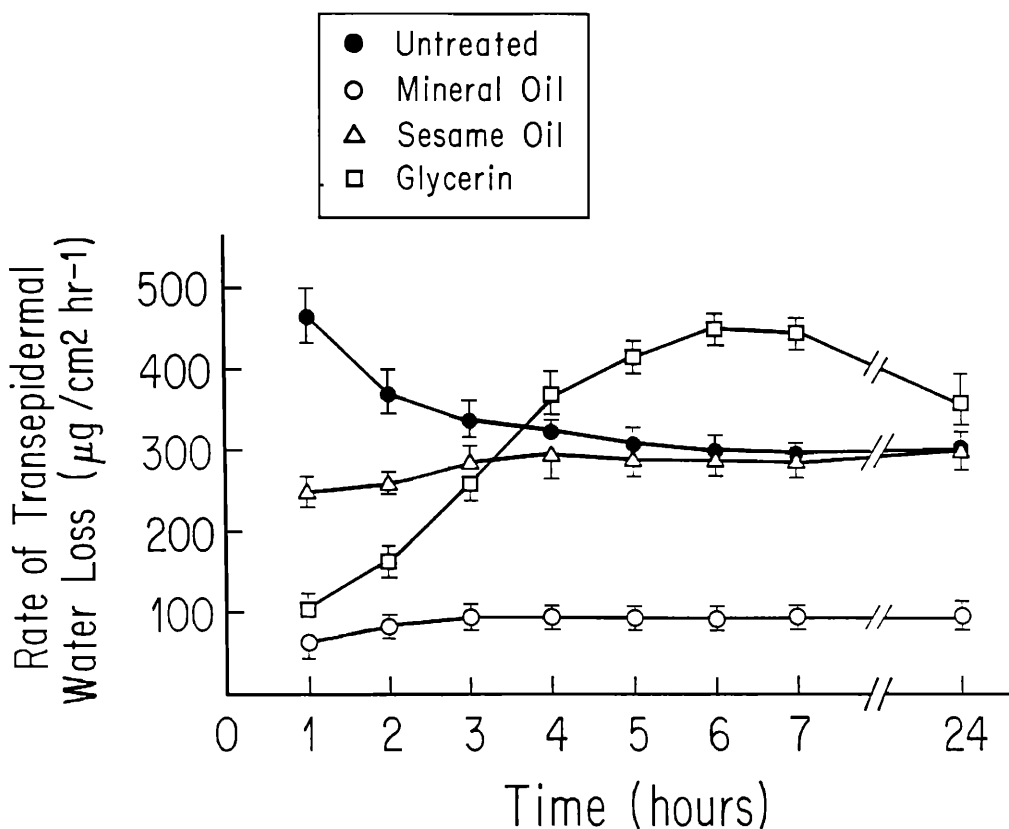


Figure 5. TEWL rates vs time values for untreated skin and skin treated with mineral oil, sesame oil, and 25% glycerin in water. Bars refer to standard error of measurement.

A two-week conditioning study was carried out with mineral oil, the most effective of the occlusive agents tested. This test provided us with some insight into the mechanism of mineral oil as a moisturizer. Results of this study are reported in Table II. No significant differences were found between treated and untreated ear skin samples. It was concluded that for mineral oil to act to decrease TEWL rate in this procedure, it must be able to bind to the skin. The protective effects of mineral oil were experimentally removed during sample preparation. Thus we conclude that mineral oil acts to decrease TEWL by imparting a physical barrier to the transport of water from the membrane. Conditioning the skin with mineral oil appeared to be an ineffective treatment modality.

CONCLUSIONS

Previously developed *in vitro* techniques for measuring TEWL are tedious and time-consuming. Most of these *in vitro* methods employ a gravimetric technique for measurement. Steady state TEWL rates cannot be achieved in less than 24 hours. Blank's *in vitro* method (30) of using tritium-labeled water to measure water loss was combined

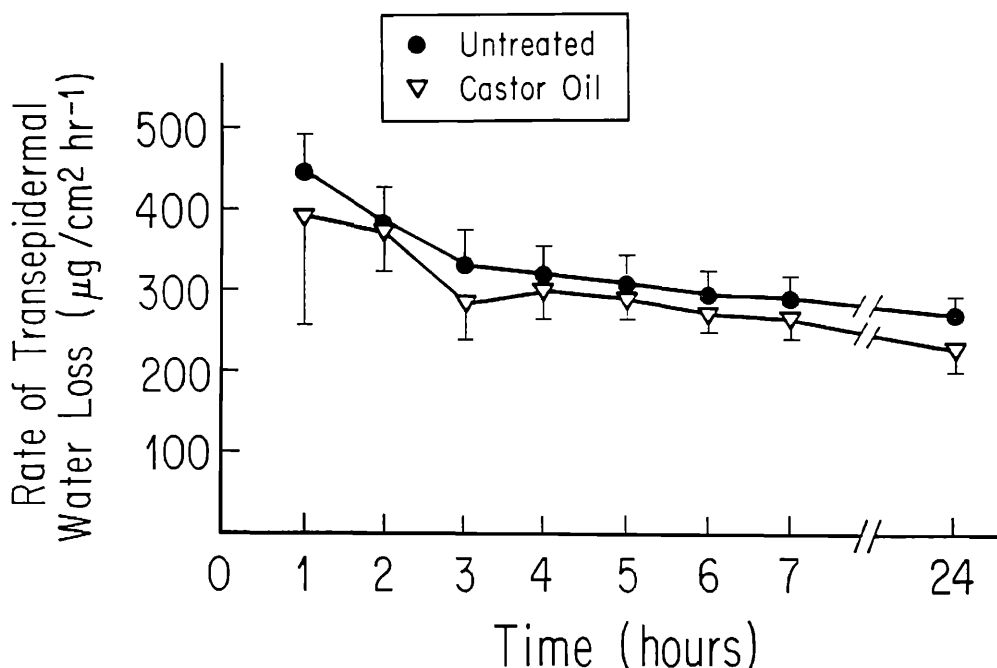


Figure 6. TEWL rates vs time values for untreated skin and skin treated with castor oil. Bars refer to standard error of measurement.

with adsorption of water onto a desiccant. The new method proved to be very efficient. TEWL equilibrium could be achieved within three hours and a full study can be completed within eight hours.

Cartilage-stripped hamster ear skin was successfully used as an *in vitro* membrane for TEWL measurement. The barrier properties of the hamster ear skin compared favorably to those of human skin. TEWL rate values agreed with values previously reported for human skin obtained under similar environmental conditions (1,2). The membrane exhibited an exponential increase in TEWL with increased temperature. A calculated energy of activation for water permeation of 13 Kcal/mole was in general agreement with the value reported previously by Scheuplein for human skin (9).

Table II
Transepidermal Water Loss Values for Mineral Oil Conditioning Ear After Various Time Periods

Time Periods:	1 hr	3 hr	6 hr	24 hr
	Treated Ear			
Mean TEWL µg/cm ² /hr	391	269	268	206
	Untreated Ear			
Mean TEWL µg/cm ² /hr	437	315	226	202
	p > 0.3	p > 0.3	p > 0.3	p > 0.3

The present *in vitro* technique potentially can also be used to evaluate the relative effectiveness of skin agents. This method has a real advantage in that the tritium-tracer technique can eliminate erroneous TEWL values that may result from water evaporation from a moisturizer. In general, it would appear that TEWL is influenced by the polarity of the agent applied to the skin. The most nonpolar of the four agents tested, namely mineral oil, showed the lowest TEWL rate. In contrast, the humectant, (25% glycerin in water)-treated skin, showed an increase in TEWL rate. Our TEWL rates for glycerin were comparable to data previously reported by Reiger and Deem (30). Occlusive agents, such as mineral oil, showed evidence of decreasing water loss by acting as a physical barrier to the transport of water through the membrane.

Further work is deemed necessary for proving the validity of this method as a pretest for moisturizer efficacy.

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