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Improved stability of the human epidermal membrane during successive permeability experiments

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Summary

In many cases it is instructive to use a single human epidermal membrane (HEM) sample to perform successive in vitro permeability experiments under varied experimental conditions. This study focused upon the feasibility of such successive permeability experiments in side-by-side, two-chamber diffusion cells. It was shown that for permeability experimental protocols which involved performing one permeability experiment per day and extensive washing between permeability experiments, the barrier properties of HEM samples were altered significantly within the first 72 h of the protocol. However, if the HEM is supported in the diffusion cell with a porous synthetic membrane, a single HEM sample remains essentially unaltered with respect to mannitol permeability and electrical resistance for up to 5 days. This suggests that protecting the HEM from physical stress is an essential element in performing successive permeability experiments.

Introduction

Over the past several decades there has been a great deal of research focused upon transdermal drug delivery (Scheuplein and Blank, 1971). Much of this research has been directed at correlating the barrier properties of the human epidermal membrane (HEM) and skin permeation data (Michaels et al., 1975). Clearly, a fundamental understanding of the barrier properties of the HEM, particularly the stratum corneum, is an essential element in developing any successful transdermal delivery system. Scheuplein pioneered much of the work aimed at characterization of the permeation pathway for molecules through the stratum corneum (Scheuplein, 1965, 1967; Blank et al., 1967). He concluded that penetration of non-electrolytes through the stratum corneum was not primarily intercellular or appendageal but was best described as penetration through a dense, effectively homogeneous phase (Scheuplein, 1965). More recently, much of the transdermal research has been directed toward iontophoresis. With this has come the characterization of the barrier properties of the stratum corneum under an applied electrical field (Burnette and Ongpipattanakul, 1987, 1988; De-

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Nuzzio and Berner, 1990; Pikal, 1990; Sims et al., 1991a, 1992; Srinivasan and Higuchi, 1991). There seems, however, to be a void in the literature pertaining to the characterization of the permeation pathway followed by ionic permeants under passive conditions. A more fundamental understanding of the barrier properties of the stratum corneum in this regard is an essential part of obtaining a mechanistic understanding of the changes which take place under an applied electric field.

One experimental obstacle to obtaining meaningful in vitro passive permeability data for HEM with ionic permeants is skin-to-skin variability (Liu et al., 1991). For this reason, it is often necessary to determine successive permeability coefficients with a single HEM sample. This technique overcomes skin-to-skin variability and allows quantitative data analysis for permeability data collected from a single HEM sample under varied experimental conditions. Blank et al. (1967) used a similar technique of performing several successive permeability experiments at different temperatures to determine the activation energies of transport for various alcohols.

Our planned first use of this technique has been to determine the effect of ionic strength of the medium on the passive transport of ionic permeants through HEM. These studies potentially will give insights into the nature of the permeation pathway of ionic permeants by revealing partitioning properties of the HEM for cations and anions; varying the ionic strength is a means by which the relative importance of the electrical double layer in the pores of the stratum corneum might be varied in a systematic manner (Sims et al., 1991b). The limitation of this method is the durability of HEM under experimental conditions.

The specific focus of this study was to establish and validate a methodology enabling the use of a single HEM sample for successive permeability experiments. It was found that HEM does maintain its initial barrier properties under experimental conditions for several days. However, when the experimental protocol involves extensive washing between permeability experiments, it is necessary to support HEM in the diffusion cell with a synthetic porous membrane. This protects the HEM from physical stress and allows it to be used for up to five days without any significant change in permeability.

Materials and Methods

Materials

Radio-labeled [^{3}H]mannitol, [^{14}C]tetraethylammonium bromide (TEAB) and [^{14}C]taurocholate were obtained from New England Nuclear, Boston, MA. Human skin was obtained from Ohio Valley Tissue and Skin Bank (Cincinnati). The epidermal membrane was removed by heat separation and immediately frozen for later use as previously described (Sims et al., 1991a). Millipore® GSWP filters (2.5 cm diameter) were obtained from Millipore Corp., Bedford, MA. Phosphate-buffered saline (PBS), pH 7.5, was prepared from reagent grade chemicals and deionized water. All buffers also contained 0.02% sodium azide.

Permeability experiments

All permeability experiments were conducted in two-chamber, side-by-side diffusion cells equipped with stirring propellers driven at 150 rpm. Both the donor and receiver volumes of these cells were 2 ml. Frozen HEM samples were thawed in 0.1 M ionic strength PBS at 4°C. The HEM was then sectioned into 2.25 cm^2 pieces and clamped between the two half-cells of the diffusion cells with the stratum corneum facing the donor chamber. The donor and receiver chambers were filled with 0.1 M PBS and the assembled cell was maintained at 37°C in a constant temperature water bath. After allowing the HEM to hydrate for 12–24 h, the cells were rinsed with fresh buffer and the receiver chamber was filled with 2 ml of 0.1 M PBS. The donor chamber was filled with buffer of the same ionic strength, premixed with the appropriate radiolabeled permeant. The permeability experiments then consisted of removing an initial sample and four additional 2 ml receiver samples over a 12 h period, receiver sample volumes being replaced with buffer after each sample. The samples were

mixed with 10 ml scintillation cocktail (Ultima Gold™, Packard, Meriden, CT) and assayed for the permeant by liquid scintillation counting using a Beckman LS 7500 liquid scintillation counter. Permeability coefficients were then determined according to the following equation,

$$P = (dQ/dt)/A \cdot C_d \quad (1)$$

where (dQ/dt) is the slope from the linear portion of a plot of cumulative amount of permeant in the receiver chamber, Q, vs time, t, A represents the diffusional area of the cell (0.75 cm^2) and C_d is the donor concentration.

Upon completion of a permeability experiment the diffusion cell was washed in preparation for the next experiment. If two consecutive permeability experiments were conducted in the same buffer, this involved four to six rinsings immediately following the permeability experiment, stirring for 10 h in the buffer then two additional rinsings before starting the next experiment. If the buffer was to be changed from 0.1 to 0.004 M ionic strength the cell was first rinsed three times with deionized water, then four times with 0.004 M PBS and stirred for 10 h in the low ionic strength PBS. The cells were then rinsed two additional times and the permeability experiments were started.

Resistance measurements

Electrical resistance of the HEM was measured when the HEM was initially mounted in the diffusion cell and periodically throughout the permeability experimental protocols. This was done by applying a small voltage (100 mV) across the HEM using a four-electrode potentiostat, which has been previously described (Masada et al., 1989), and measuring the resulting current. Resistance was then calculated according to Ohm's law. Although the experimental protocols studied often involved changing the ionic strength of the medium between experiments, resistance measurements were always taken during stages of the permeability protocols when the cell contained 0.1 M PBS. Resistance measurements made it possible to prescreen HEM samples and monitor the stability of the HEM throughout the various experimental protocols studied (Kasting and Bowman, 1990).

Supporting the HEM

In an attempt to extend the length of time which a HEM sample could be used for permeability experiments, a Millipore® GSWP filter was placed next to the HEM between the two half-cells of the diffusion cells. The filters were intended to relieve physical stress from the HEM which results from sampling of the cell and washing between permeability experiments. The permeability of these filters is high enough that they do not significantly contribute to the permeability of the HEM.

Dual-permeant permeability experiments

To validate the effectiveness of the supported HEM in determining successive permeability coefficients, permeability experiments were carried out simultaneously for two permeants. Both [^{3}H]mannitol and [^{14}C]TEAB were placed in the donor solution. The receiver samples from these experiments were assayed for each permeant using a Packard Tri-Carb™ 2500TR Liquid Scintillation Analyzer.

Results and Discussion

Permeability experiments with unsupported HEM

Several successive permeability experiments were conducted with TEA, taurocholate and mannitol as the permeants. The initial objective was to investigate the effect of ionic strength of the medium on the passive permeation of ionic permeants through HEM. The experimental protocol of choice involved determining three permeability coefficients for a single HEM sample. The first and third permeability coefficients were to be determined in the same ionic strength medium to insure that the HEM sample remained unchanged during the experiment. The second permeability coefficient was to be determined in a medium of different ionic strength than the first/third. The strength of this procedure is based upon being able to attribute any differences in the second permeability coefficient

from the first and third to the effect of ionic strength as long as the first and third permeability coefficients remain constant.

Table 1 shows both the resistance measurements and the permeability coefficients for a set of 12 HEM samples. Each run constitutes the successive permeability coefficients determined for a specific HEM sample. The permeants for this set of experiments were TEA (runs 1–6) and taurocholate (runs 7–12). The ionic strength at which the permeability coefficients were determined is indicated and the resistance measurements were taken after the HEM had equilibrated in 0.1 M PBS. The limitation of this procedure was that, as can be seen from Table 1, the permeability coefficients generally increase from the first to the third. Resistance measurements also show a general decrease in the resistance of the HEM samples from day 1 to day 3. It is important to note that in the cases where the resistance remained relatively constant, the first and third permeability coefficients are also quite comparable. It is also interesting to note that in all except one of the HEM samples, there seems to be an ionic strength dependent trend. This trend shows an increase in permeability for the cationic TEA with decreasing ionic strength and a decrease in permeability for the anionic taurocholate with decreasing ionic strength. The difficulty in quantitatively analyzing this data is that the trend is softened by the alteration of the membrane which is apparent both from drifting resistance measurements and drifting permeability coefficients from day 1 to day 3.

Figs 1 and 2 show both the resistance data and permeability coefficients for two representative mannitol permeability experiments. Fig. 1 represents a 3 day protocol in which permeability coefficients are determined on the first and third day. The ionic strength remained constant, therefore the sample did not have to undergo as rigorous a washing protocol as when the ionic strength is changed. However, by the third day the electrical resistance had decreased by almost a factor of two and the permeability had increased by almost a factor of two. Fig. 2 shows the data from a mannitol protocol which involved changing the ionic strength from 0.1 to 0.004 M after day 1 and

TABLE 1

Resistance values and successive permeability coefficients determined for TEA and taurocholate

HEM sample no.	Resistance ($k\Omega\ cm^2$)			Permeability (cm/s) ($\times 10^8$)		
	R_1	R_2	R_3	P_1	P_2	P_3
TEA						
1 [a]	13.4	5.2	4.1	5.5	27.6	37.4
2 [a]	6.2	5.9	6.4	21.8	43.0	22.8
3 [a]	8.5	7.3	3.8	8.8	46.7	52.3
4 [b]	8.3	9.3	9.5	37.1	14.3	24.5
5 [b]	14.7	9.6	6.0	35.7	9.6	59.1
6 [b]	15.1	7.3	3.0	34.9	11.4	70.9
Taurocholate						
7 [c]	4.9	2.4	1.8	2.4	1.2	16.6
8 [c]	3.9	1.4	0.8	1.2	1.0	27.1
9 [c]	10.6	7.3	6.7	1.8	0.8	3.3
10 [d]	12.5	11.0	7.0	0.7	2.0	0.7
11 [d]	25.5	19.0	7.7	1.2	1.7	1.4
12 [d]	51.7	58.7	38.6	0.23	0.37	0.28

[a] Runs 1–3: 0.1 to 0.004 to 0.1 M.
[b] Runs 4–6: 0.004 to 0.1 to 0.004 M.
[c] Runs 7–9: 0.1 to 0.004 to 0.1 M.
[d] Runs 10–12: 0.004 to 0.1 to 0.004 M.

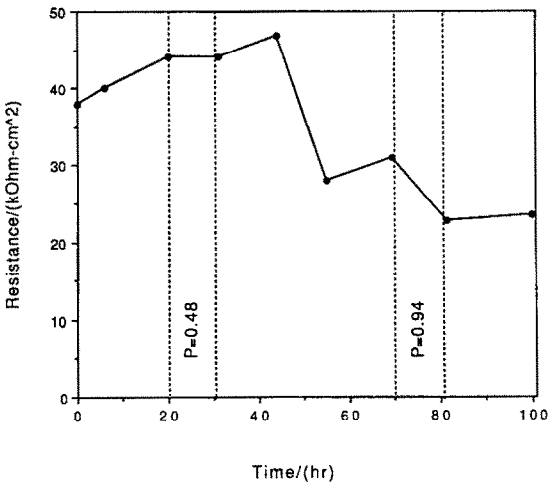

Fig. 1. Mannitol permeability experiment (3 day protocol) with unsupported HEM. Permeability coefficients ($\times 10^8$ cm/s) shown in time interval which they were determined. Ionic strength remained at 0.1 M and resistance time course shown by line graph.

back to 0.1 M after day 2. In this case the permeability again increased coupled with a decreasing resistance.

Permeability experiments with supported HEM

Figs 3 and 4 present data from representative mannitol permeability experiments in which the

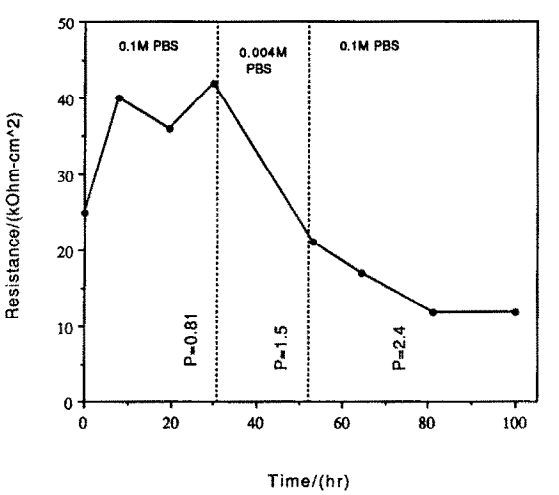

Fig. 2. Mannitol permeability experiment (3 day protocol) with unsupported HEM. Permeability coefficients ($\times 10^8$ cm/s) shown in time interval which they were determined. Ionic strength varied as indicated and resistance time course shown by line graph.

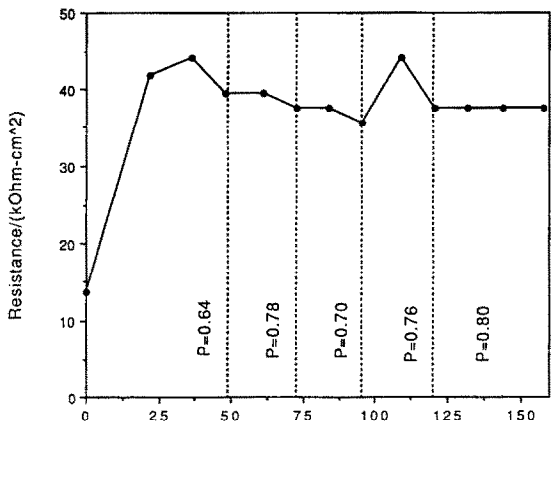

Fig. 3. Mannitol permeability experiment (5 day protocol) with supported HEM. Permeability coefficients ($\times 10^8$ cm/s) shown in time interval which they were determined. Ionic strength remained at 0.1 M and resistance time course shown by line graph.

HEM had been supported by a Millipore® filter. Each of the permeability coefficients in Fig. 3 were determined in 0.1 M PBS. As can be seen from Fig. 3, both the resistance and the permeability coefficients remained constant throughout

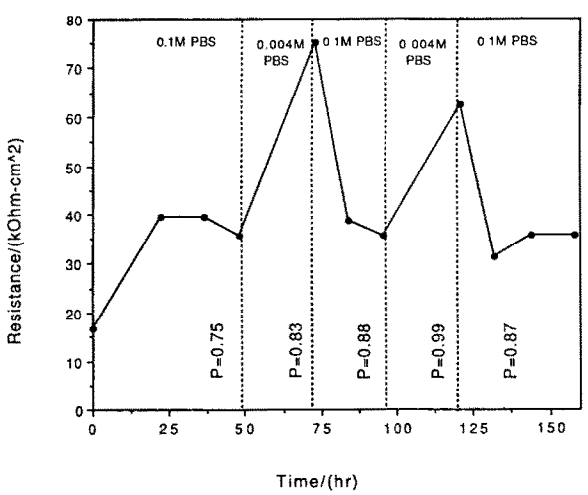

Fig. 4. Mannitol permeability experiment (5 day protocol) with supported HEM. Permeability coefficients ($\times 10^8$ cm/s) shown in time interval which they were determined. Ionic strength varied as indicated and resistance time course shown by line graph.

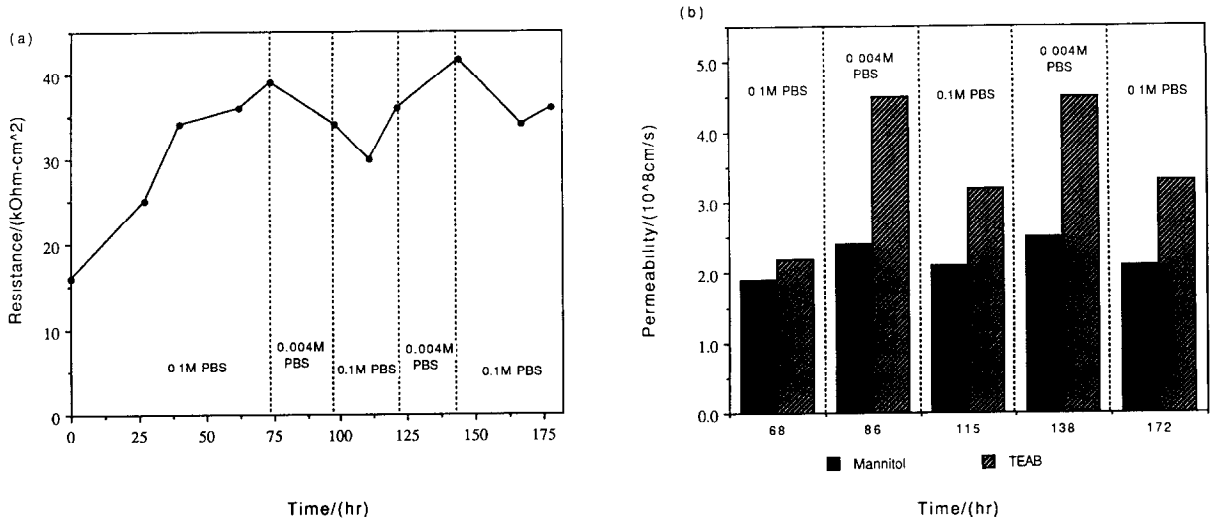

Fig. 5. (a) Resistance time course for 5 day dual-labeled permeability experiments with supported HEM. Ionic strengths varied as indicated. (b) Permeability coefficients determined simultaneously for TEA (hatched bars) and mannitol (filled bars). Times indicated are midpoints of permeability experiments corresponding to the time axis of panel a.

the 5 day protocol. Fig. 4 shows data from a protocol where the ionic strength was alternated from 0.1 to 0.004 M and vice versa. Again the permeability remained constant throughout the 5 day protocol. The resistance of the HEM sample is dependent upon the changes in ionic strength of the medium as one would expect. Immediately following the change from low to high ionic strength, the resistance of the HEM is higher than at its stable level in the high ionic strength media. Within approx. 3–6 h after the ionic strength change the resistance returns to a value similar to that measured prior to the ionic strength change.

Dual-permeant permeability experiments

The advantage of supporting the HEM during the ionic strength experimental protocol is illus-

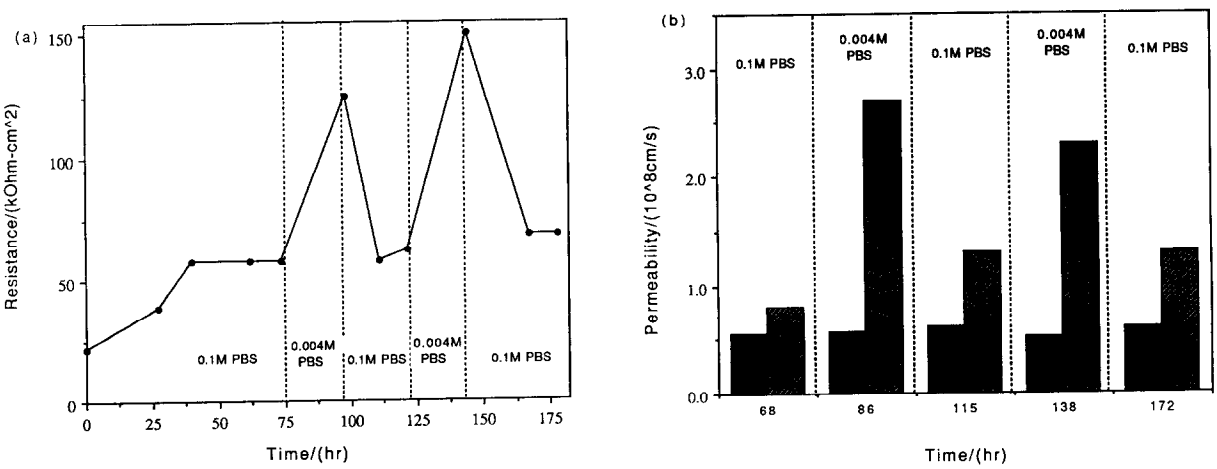

Fig. 6. (a) Resistance time course for 5 day dual-labeled permeability experiment with supported HEM. Ionic strength varied as indicated. (b) Permeability coefficients determined simultaneously for TEA (hatched bars) and mannitol (filled bars). Times indicated are midpoints of permeability experiments corresponding to the time axis of panel a.

trated by Figs 5a,b and 6a,b. These figures show the permeability coefficients determined simultaneously for TEA and mannitol as well as the resistance data for the five day protocol. The ionic strength effect upon the permeability of TEA is clear in these experiments and follows the same trend indicated by the data in Table 1. Both the resistance and the permeation of the neutral permeant, mannitol, are constant for these HEM samples. This makes the assignment of the change in TEA permeability to ionic strength effects straightforward.

Conclusion

It has been shown that supported HEM can maintain its initial permeability properties under experimental conditions for five days. It has also been demonstrated that this allows for experimental protocols which add a quantitative nature to transdermal permeation and iontophoresis studies. By performing successive permeability experiments with a single HEM sample a number of experimental variables can be investigated without the difficulty of factoring out skin-to-skin variability. Future applications of this method include the coupling of ionic strength dependent partitioning of ionic permeants in HEM with electro-osmotic data (Sims et al., 1991b) and/or hindered diffusion experiments (Beck and Schultz, 1972). This will enable the estimation of effective 'pore size' and surface charge density of the 'pore wall' for HEM samples.

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