Iontophoretic Permselectivity of Mammalian Skin: Characterization of Hairless Mouse and Porcine Membrane Models

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Purpose. To evaluate the transport number of Na^+, and the isoelectric point, of two skin membranes frequently used for iontophoretic *in vitro* research.

Methods. Na^+ transport numbers were determined by the Hittorf method or by the measurement of membrane potential. The skin isoelectric point was deduced from the electroosmosis of mannitol (a polar non-electrolyte) as a function of pH.

Results. The Na^+ transport number across porcine skin, like that for hairless mouse, indicated a modest cation permselectivity. Consistent with this observation, the isoelectric points of porcine and hairless mouse skin were determined to be in the ranges of 3.5–3.75 and 4.5–4.6, respectively. That is, at physiological pH, both of these model membranes supports a net negative charge.

Conclusions. The permselective properties of porcine and hairless mouse skin are similar (but with the porcine membrane having apparently fewer basic or more weakly-acidic groups than that of the mouse) and consistent with the characteristics, which have been deduced elsewhere, of human skin.

KEY WORDS: iontophoresis; electroosmosis; transdermal delivery; mannitol; permselectivity; skin isoelectric point.

INTRODUCTION

The relative contributions of electrorepulsion and convective solvent flow to transdermal iontophoretic transport remain ill-defined. Unambiguous experiments, which can separate the roles of these two mechanisms, have proved difficult to design and implement. The situation is complex (and therefore of considerable interest) because the skin, under normal conditions, itself supports a charge and because the importance of electrorepulsion and electroosmosis depends upon the properties (such as, in particular, the molecular size) of the permeating species. The relevance of this observation to the feasibility of iontophoretic drug delivery has not escaped the attention of pharmaceutical scientists in the field (1–3).

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Convective solvent flow consists mostly of electroosmosis, which is a consequence of the net (normally negative) charge on the membrane, and a resultant permselectivity to counterion movement. Small contributions from so-called transport and hydration number effects (≤5%) have also been identified (1). Qualitatively speaking, it is reasonable to posit that electroosmosis is a second-order phenomenon when the transport number of the ion of interest is high—under these circumstances, electrorepulsion dominates mechanistically. On the other hand, for ions which do not carry a significant fraction of the total charge flowing across the skin (e.g., for higher molecular weight species), electroosmosis assumes a much greater significance. It follows that an important objective is to assign a quantitative aspect to the preceding statements.

Characterization of the skin's permselective properties is essential to achieve the long-term goals of mechanistic understanding and optimization of iontophoretic drug delivery. The focus of this and recent work (2,4–7) from our laboratories is the role of electroosmosis, which clearly depends upon the net charge on the skin (and therefore the pH of the solutions in contact with the membrane) and the resulting transport numbers of the predominant ion species present (in particular, Na^+). Thus, the specific aims of the experiments reported in this paper were (a) to evaluate the transport number of Na^+, and (b) to determine the isoelectric point of two membranes commonly used in *in vitro* iontophoretic studies.

MATERIALS AND METHODS

Materials

N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) and D-mannitol were purchased from both Aldrich Chemical Co. (Milwaukee, WI) and Sigma Aldrich Química SA (Madrid, Spain). ^{14}C-Mannitol (50–60 mCi/mmol) was obtained from either Moravek Biochemicals (Brea, CA) or Amersham Ibérica, SA (Madrid, Spain). All other chemicals were at least analytical grade.

Skin

Three skin membranes were used in the different experiments performed: (a) full-thickness skin was excised from 8–12 weeks old female hairless mice (HRS/hr hr, Simonsen Laboratories, Gilroy, CA) immediately after euthanasia by CO_2 asphyxiation; (b) full-thickness skin, excised post-sacrifice, and either used at once or frozen until use, from neonatal (2–6 days old) pigs (J. Bermúdez-García, Santiago, Spain); and (c) heat separated epidermis from human cadaver stored frozen until use.

Transport Number Experiments

Measurements were made using either human epidermis or fresh or frozen full-thickness pig skin. The transport number of Na^+ across the different barriers was determined by either the Hittorf method (4,8–10) or by the measurement of the membrane potential (4,10–12). In the former approach, skin was clamped between the two identical halves of a standard side-by-side diffusion cell (Crown BioScientific, Inc., Somerville, NJ). The chambers on either side of the skin were filled

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with 154 mM NaCl (aq.), the pH of which was adjusted to 7.4 by addition of 1M NaOH. After 1 hour of equilibration, fresh solutions were introduced, together with Ag/AgCl electrodes, prepared in the usual way (13) and connected to a constant current source (Kepco Power Supply APH 1000 M, Kepco Inc., Flushing, NY). Constant current (0.64 mA/cm^2) was passed between the electrodes for 3 hours, at the end of which the change in osmolality, and hence the transport number of Na^+, was assessed (Osmomat 030, Gonotec, Berlin, Germany) (4).

For the measurement of membrane potential (V_m), the skin was mounted as before in side-by-side diffusion cells thermostated at 37°C. The chamber facing the epidermal surface was filled with 1 M NaCl, while the solution bathing the dermal side was 0.1 M NaCl (both solutions were adjusted to pH 7.4 as before). After equilibration at 37°C, Ag/AgCl electrodes were introduced into the chambers and the electromotive force (e.m.f.) was measured (Digital Multimeter TES 2360, TES Electronic Corp., Barcelona, Spain). This value was corrected to V_m by subtraction of the electrode potential which was determined in essentially the same experimental set-up in the absence of skin (in this case the solutions of different NaCl concentrations were separated by a 3% agarose salt bridge containing 1M KCl). The Na^+ transport number (t_{Na^+}) was then calculated from the equation (4,11):

$$t_{Na^+} = 0.5 + (F \cdot V_m)/[2RT\ln (C_2/C_1)]$$

where F is the Faraday constant (96500 C/mol), C_2 = 1 M; C_1 = 0.1 M; R is the universal gas constant and T is the absolute temperature.

Skin Isoelectric Point Determination

To evaluate the isoelectric point of the skin, electroosmosis across the membrane was measured by following the transport of the polar, non-electrolyte mannitol (4,5) under a variety of pH conditions. These experiments were performed with either hairless mouse or porcine skin (insufficient human samples were available). The tissue was clamped between the two halves of conventional side-by-side diffusion cells (Crown Bioscientific, Inc., Somerville, NJ). The donor phase always faced the epidermal side of the skin and held either the positive or negative electrode. This compartment contained 1 mM mannitol, "spiked" with the ^{14}C-radiolabeled sugar (1 μCi/cell), dissolved in 3 mL of 133 mM NaCl and buffered with 25 mM HEPES to one of four specific pH values (see below). The receptor compartment, which held the electrode of opposite charge, contained the identical buffered electrolyte (without mannitol) and was sampled regularly for the permeated radio-marker by manually removing an aliquot of the solution (which was immediately replenished with fresh buffer).

The experiments were performed in side-by-side diffusion cells under symmetrical pH conditions. In the experiments with hairless mouse skin, 0.4 mA total current (corresponding to a current density of 0.51 mA/cm^2) was passed for 4 hours, and anodal and cathodal mannitol transport was determined at pH 4.0, 4.5, 4.65, and 5.0. For neonatal pig skin, a slightly higher current (0.5 mA; 0.64 mA/cm^2) was used, and the pH values investigated were 3.5, 3.75, 4.0, and 7.4. Current was delivered to the Ag/AgCl electrodes from a power supply providing constant current (Kepco Power Supply APH 1000 M, Kepco Inc., Flushing, NY).

Table I. Values of t_{Na^+} Determined in this Work

Skin	Method	t_{Na^+} (mean ± SD)	n[a]
Porcine, frozen	e.m.f.	0.58 ± 0.15[b]	12
Porcine, fresh	e.m.f.	0.53 ± 0.11[b]	9
Porcine, fresh	Hittorf	0.46 ± 0.13[b, c]	12
Human, frozen[d]	Hittorf	0.39 ± 0.04[e]	9

[a] Number of replicate measurements; skin was obtained from two different pigs and from a single human cadaver.
[b] Not significally different.
[c] Value determined from measurements at the anode only (see text).
[d] Heat-separated epidermis.
[e] Average of values determined at the anode (0.38 ± 0.02) and at the cathode (0.40 ± 0.06).

The receptor phase samples were analyzed for ^{14}C-mannitol by liquid scintillation counting (Ready Gel; Liquid Scintillation Counter LS 6000 LL, Beckman Instruments Inc., Fullerton, CA) and the dpm values were converted to molar flux. All measurements were made in at least triplicate and the data were compared by appropriate statistical tests as described below.

RESULTS AND DISCUSSION

The Na^+ transport numbers determined in this work are in Table I. The e.m.f. method revealed that t_{Na^+} through frozen porcine skin was statistically indistinguishable from that in fresh tissue. The values suggest a modest cation permselectivity of the membrane, not inconsistent with previous t_{Na^+} measurements using other skin membranes (Table II). The estimation of t_{Na^+} from changes in osmolality at the anode (i.e., using the Hittorf method) was consistent with the e.m.f. data. However,

Table II. Previously Determined Values of t_{Na^+}

Skin	Method	Electrolyte, buffer, pH, etc.	t_{Na^+}	Ref.
human cadaver (fresh)	Hittorf $^{22}Na^+$ 0.08–0.23 mA/cm^2	25 mM Hepes &133 mM NaCla; pH 7.4	0.62	9
human skin bank (frozen)	e.m.f.	NaCl Gradient 10:1	0.60	12
human cadaver (frozen)	Hittorf; $^{22}Na^+$; 0.05 mA/cm^2	Dulbecco - PBS pH 7.4	0.51	8
human cadaver (frozen)	Hittorf $^{22}Na^+$; 0.05 mA/cm^2	Dulbecco - PBS pH 4.0	0.24	8
human cadaver (fresh)	Hittorf $^{22}Na^+$; 0.05 mA/cm^2	Dulbecco - PBS pH 7.4	0.60	14
hairless mouse	Hittorf; osmolality 0.51 mA/cm^2	133 mM NaCl, pH 7.4	0.46	4
hairless mouse	e.m.f.	NaCl 200/100 mM	0.43	4
hairless mouse (stripped)	Hittorf; osmolality 0.51 mA/cm^2	133 mM NaCl, pH 7.4	0.47	4
hairless mouse	Hittorf 1.6 mA/cm^2	50 mM NaCl pH 6.0	0.33	11
hairless mouse	e.m.f.	NaCl Gradient 2:1	0.39	11

the results from the cathode were quite divergent (0.28 ± 0.05; n = 12) due, we believe, to the leakage of endogenous ions from the formerly viable layers (living epidermis + dermis) of the skin. The Hittorf procedure assumes that the measured changes in osmolality result only from changes in Na^+ and Cl^- concentrations due to the associated electrochemical and iontophoretic processes taking place. The participation of other species created an artificial response, therefore. Because the anode was positioned on the stratum corneum side of the skin, the "contamination" of the readings by skin-associated ions was probably close to negligible; at the cathode, on the other hand, which was located on the membrane's dermal side, the movement of skin associated ions into the bathing electrolyte is much more likely.

The t_{Na^+} of human skin determined by the Hittorf procedure was 0.39 ± 0.04, a value somewhat less than those which have been reported in the literature (Table II). In this case, however, replicate measurements were possible using skin from only one donor, and the anode-cathode asymmetry was not observed, probably because the heat-separation procedure, by which the epidermis was obtained, removed in large part, the endogenous ions of the skin.

The anodal and cathodal fluxes of mannitol, after 4 hours of iontophoresis as a function of pH, are presented in Figures 1 and 2 for hairless mouse skin and neonatal porcine skin, respectively. Because of the slightly different current densities used for the two membranes, the results have been normalized by this parameter. It is immediately apparent from these data that electroosmosis, and hence the permselectivity and the net charge of the skin, are sensitive to pH. For both membranes used, as pH is decreased, there is a point at which the anodal and cathodal fluxes become esentially identical such that one may define an apparent isolectric point (pI) of the tissue. For hairless mouse skin the pI ≈ 4.5–4.6, while for neonatal porcine skin it is lower, pI ≈ 3.5–3.75.

Closer inspection of the flux values (Table III) reveals additional details. For hairless mouse skin, comparison of anodal and cathodal fluxes at each pH value using the Mann-Whitney Rank Sum Test revealed that cathodal transport was significantly greater than anodal at pHs 4.0 and 4.50 ($p < 0.01$); at pH 5.0, on the other hand, anodal was greater than cathodal ($p < 0.01$), whereas at pH 4.65 there was no statistical difference. When anodal fluxes alone were evaluated, ANOVA followed by the Student-Newman-Keuls test indicated that the values at pHs 4.0 and 4.5 were significantly different ($p < 0.05$) from those at pH 4.65 and 5.0. From the cathode, electroosmosis was less sensitive to pH: the Kruskall-Wallis test, followed by Dunn's method, showed that transport at pHs 4.0, 4.50, and 4.65 were indistinguishable, as were the fluxes at pH 4.50, 4.65, and 5.0. Taken together, these findings are fully consistent with the cationic permselectivity of hairless mouse skin at pH values above 5.0 and a pI of the membrane between 4.5 and 4.6; at pH < pI, the membrane shows a marked anion permselectivity and must support, therefore, a net positive charge.

For neonatal porcine skin, the Mann-Whitney Rank Sum Test at pHs 7.4 and 4.0 showed that anodal transport was significantly greater than cathodal ($p < 0.001$ at pH 7.4, $p < 0.05$ at pH 4.0); no statistical differences were detected, however, between anodal and cathodal fluxes at pHs 3.75 and 3.50. Parenthetically, though, it should be noted that after 8 hours of iontophoresis, rather than 4, cathodal flux had become more important ($p < 0.05$) than anodal at pH 3.50; by contrast, no difference remained between anode and cathode at pH 3.75.

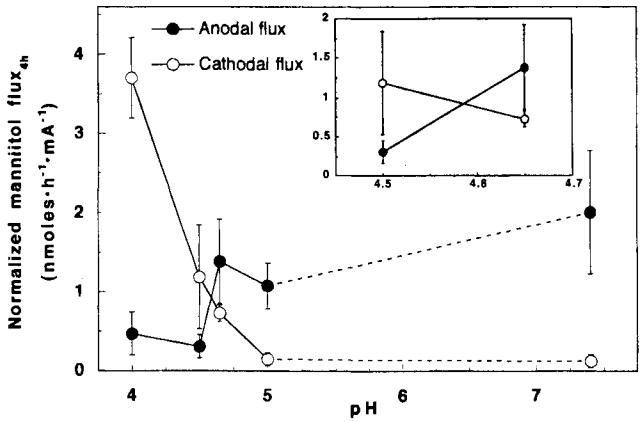

Fig. 1. Anodal (●) and cathodal (○) mannitol fluxes through hairless mouse skin as a function of the pH. Values at pH 7.4 are from reference (5). Each data point represents the mean (±SD) of 3–6 determinations.

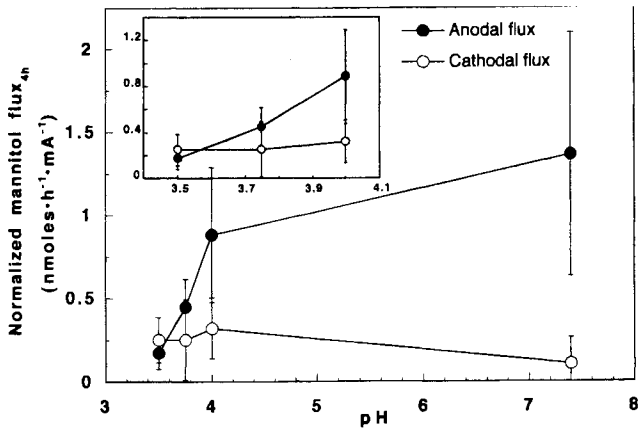

Fig. 2. Anodal (●) and cathodal (○) mannitol fluxes through neonatal porcine skin as a function of pH. Each data point represents the mean (±SD) of 5–7 determinations.

Table III. Iontophoretic Fluxes of Mannitol Across Hairless Mouse and Neonatal Porcine Skin as a Function of pH

Skin[a]	pH[b]	Anodal Flux[c]	Cathodal Flux[c]
Hairless Mouse	7.40	2.01 ± 0.78[d]	0.12 ± 0.08[d]
	5.00	1.08 ± 0.29	0.15 ± 0.08
	4.65	1.38 ± 0.53	0.73 ± 0.10
	4.50	0.31 ± 0.15	1.19 ± 0.66
	4.00	0.47 ± 0.27	3.70 ± 0.51
Porcine	7.40	1.36 ± 0.73	0.11 ± 0.16
	4.00	0.88 ± 0.41	0.32 ± 0.18
	3.75	0.45 ± 0.16	0.25 ± 0.24
	3.50	0.17 ± 0.10	0.25 ± 0.14

[a] Full-thickness tissue.
[b] pH of both donor and receptor solutions.
[c] Units are $nmol \cdot cm^{-2} \cdot h^{-1} \cdot (mA \cdot cm^{-2})^{-1}$. Values determined after 4 hours of iontophoresis (mean ± SD; n ≥ 3).
[d] Previously published values (5).

The anodal fluxes, when compared by Kruskall-Wallis and then Dunn's method, showed only a weak pH dependence: the values at pHs 7.4, 4.0, and 3.75 were not statistically distinguishable, nor were those at pH 3.75 and 3.5. From the cathode, Kruskall-Wallis revealed no differences in flux as a function of pH. Again these results are consistent with a cation-selective membrane at physiological pH and an isolectric point less than 4 (we estimate the pI to be 3.75–3.5). However, unlike hairless mouse skin, which reverses its behavior to become quite permselective to anions at pH 4, neonatal porcine skin has not reached the point of neutralization at this concentration of protons. This would appear to reflect a very real difference in the compositional nature of the iontophoretic pathways through the two skin models (i.e., the presence of fewer basic or a greater number of weakly acidic groups in neonatal porcine skin than in hairless mouse skin).

More detailed interpretation of the findings from this work is not possible from the data obtained. Practically speaking, a logical next step is to confirm that human skin behaves in a similar way (as might be reasonably expected); however, with respect to drug delivery, it seems that manipulation of the electrode chamber pH is likely to be of limited use with respect to alteration of the skin's iontophoretic barrier properties. To elicit a dramatic effect, the pH would have to be changed to an extent that serious concerns about skin irritation and tolerability would inevitably arise.

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REFERENCES

1. M. J. Pikal. The role of electroosmotic flow in transdermal iontophoresis. *Adv. Drug Del. Rev.* **9**:201–237 (1992).
2. J. Hirvonen, Y. Kalia, and R. H. Guy. Transdermal delivery of peptides by iontophoresis. *Nature Biotechnology.* **14**:1710–1713 (1996).
3. P. Green. Iontophoretic delivery of peptide drugs. *J. Control. Rel.* **41**:33–48 (1996).
4. A. Kim, P. G. Green, G. Rao, and R. H. Guy. Convective solvent flow during iontophoresis. *Pharm. Res.* **10**:1315–1320 (1993).
5. M. B. Delgado-Charro and R. H. Guy. Characterization of convective solvent flow during iontophoresis. *Pharm. Res* **11**:929–935 (1994).
6. P. Santi and R. H. Guy. Reverse iontophoresis-Parameters determining electroosmotic flow: I. pH and ionic strength. *J. Control. Rel.* **38**:159–165 (1996).
7. P. Santi and R. H. Guy. Reverse iontophoresis-parameters determining electro-osmotic flow. II. Electrode chamber formulation. *J. Control. Rel.* **42**:29–36 (1996).
8. G. B. Kasting and L. A. Bowman. DC electrical properties of frozen, excised human skin. *Pharm. Res.* **7**:134–143 (1990).
9. R. R. Burnette and B. Ongipipattanakul. Characterization of the permselective properties of excised human skin during iontophoresis. *J. Pharm. Sci.* **76**:765–773 (1987).
10. F. Helfferich. Ion Exchange, Dover Publications, Inc., New York, 1995.
11. M. J. Pikal and S. Shah. Transport mechanisms in iontophoresis. II. Electroosmotic flow and transference number measurements for hairless mouse skin. *Pharm. Res.* **7**:213–221 (1990).
12. J. D. DeNuzzio and B. Berner. Electrochemical and iontophoretic studies of human skin. *J. Control. Rel.* **11**:105–112 (1990).
13. P. G. Green, R. S. Hinz, C. Cullander, G. Yamane, and R. H. Guy. Iontophoretic delivery of amino acids and aminoacid derivatives across the skin *in vitro*. *Pharm. Res.* **8**:1113–1120 (1991).
14. G. B. Kasting and L. A. Bowman. Electrical analysis of fresh, excised human skin: A comparison with frozen skin. *Pharm. Res.* **7**:1141–1146 (1990).