Sites of Iontophoretic Current Flow Into the Skin: Identification and Characterization with the Vibrating Probe Electrode

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The routes taken by charged substances (e.g., peptides) through the skin during iontophoretic drug delivery are not well characterized. We have used a vibrating probe electrode to reproducibly identify and vectorize site-specific (spatial resolution = 20 μm) ionic flows as they were occurring in hairless mouse skin clamped at clinically relevant current densities. These iontophoretic currents were primarily appendageal, and certain appendages (e.g., small hairs) appeared to carry most of the current. This finding may have important ramifications with respect to irritation, allergic reaction, and electrical current damage in iontophoretic drug delivery. The size and direction of the current vectors could change under certain conditions (e.g., in an unbuffered preparation, where pH changes occurred during the experiment). The vibrating probe can operate in (and is not adversely affected by) the ranges of pH, toxicity, and current required for the study of iontophoretic currents. J Invest Dermatol 97:55–64, 1991

The transdermal penetration of ionizable drugs can be enhanced by the use of an electrical potential to drive the charged molecules through the permeability barrier of the skin [1]. The factors influencing this electrically facilitated transport (iontophoresis) remain poorly understood. In particular, the routes taken by charged substances through the epidermal barrier of mammalian skin have not been adequately characterized [2]. The localization of the paths of current flow in this heterogeneous epithelium represents an intriguing scientific question per se, as well as an objective potentially relevant to the therapeutic delivery of ionizable drugs through the skin.

There is evidence that transdermal iontophoretic transport occurs primarily at discrete sites, or “pores” [2–7], although in some cases diffuse permeation may also occur [8,9]. The studies cited above have limited utility, however, because (a) the large dyes used to localize the pores are unlikely to be good model compounds for the permeation behavior of small molecules; and (b) the dye pattern formed is a static image, and thus provides no information about changes in flow magnitudes or permeation routes that may occur during the procedure, if, for example, the imposed current level or the pH changes. Such real-time information can, however, be obtained using electrophysiologic techniques such as the vibrating probe electrode [10].

The vibrating probe approach measures slowly changing extracellular electrical currents. The probe has a spatial resolution of 5–40 μm, and currents very near the surface of a tissue can be located in real time. The method is known for its low noise and low drift, and has been used to study transport pathways in amphibian epithelia [11–17], as well as currents around epithelial cell cultures [18]. A short examination of the operation and use of the probe is given below.

We have used a vibrating probe electrode to identify and vectorG5ize (i.e., determine magnitude and direction of) iontophoretic currents in a mammalian skin preparation. In particular, we have been able to demonstrate that the probe can reproducibly quantify site-specific ionic flows that occur when a clinically relevant current density is maintained across the skin, and that—in mouse skin, at least—(a) these iontophoretic currents are primarily appendageal; (b) certain appendages (e.g., small hairs) appear to carry more current than others (e.g., large hairs); and (c) the magnitude and direction of these iontophoretic currents can change under certain circumstances (e.g., in an unbuffered preparation, or when the current density is large). The probe can operate in (and is not adversely affected by) the ranges of pH, toxicity, and current required for the study of iontophoretic currents. The experimental geometry and conditions used permitted good spatial and temporal resolution.
Figure 1. The vibrating probe electrode is a metal needle with a tiny ball of platinum black at its tip (top). When the electrode is vibrated up and down in a potential gradient it "sees" a sinusoidal signal (bottom).

Overall, therefore, we describe a procedure that shows much potential for mechanistically investigating iontophoretic drug delivery.

MATERIALS AND METHODS

**Tissue** Female hairless mice (HRS/hr hr, 6–8 weeks old, Simon- sen Labs, Gilroy, CA) were sacrificed by CO₂ euthanasia. Subcutaneous fat was removed from the full-thickness dorsal skin, which was then spread out (epidermis up) on a dissection tray and pinned down on plastic film laid over the wax. Residual hairs were carefully clipped short with a small electric shaver (Model BS-1, Clairoir Inc., Stamford, CT) to prevent them from tangling with or damaging the probe. Lucite "washers" (OD = 2.54 cm, ID = 1.58 cm, area = 1.98 cm², thickness = 0.158 cm) were then cemented to the epidermis with cyanocrylate cement (Krazy Glue, Krazy Glue Inc., Itasca, IL), and any excess skin was trimmed from the outer edge of the washer. This procedure kept the skin sample flat, and prevented it from wrinkling or bunching when it was inserted into the experimental chamber. The sample was then placed onto filter paper (9 cm, Whatman #5, Fisher, San Francisco, CA) soaked with the saline solution to be used in the experiment, and the center of the washer was filled with the same solution. The preparations were refrigerated at 4°C and allowed to hydrate [6,19] in a covered plastic petri dish (100 x 15 mm, Fisher, San Francisco, CA) for a minimum of 12 h before use.

**Vibrating Probe: Principles** Although the principles and operation of the vibrating probe electrode have been described in detail elsewhere [11–13], a brief review of the technique is in order here. For the sake of simplicity, the following describes the operation of a probe measuring currents in one dimensional only. The vibrating probe is a method used to measure steady or slowly varying current coming out of a cell or tissue that is surrounded by a conductive medium (e.g., saline solution). This current results in a voltage drop in the medium, the size of which is proportional to the resistivity of the solution. The probe measures this voltage drop. It can operate as close as 5 μm to the tissue, but is usually kept at 4–5 times this distance to avoid damage to the tip. Because current (I) is related to voltage (V) and resistance (R) by Ohm’s Law (V = IR), the measured voltage can be equated to current density (current per unit area) if one knows the resistivity of the local solution. Note, however, that the best estimate of the actual current density flowing through the tissue will be obtained with the probe relatively close to the surface, because as it moves farther away the measurement can be “contaminated” by currents from adjacent areas.

The probe itself is a platinum-iridium needle that is insulated except at its tip, where there is a tiny (5–20-μm diameter) sphere of platinum black (Fig 1, top). It is thus a metal electrode (rather than a metal/metal salt electrode, such as Ag/AgCl). Metal electrodes are very good for measuring high frequencies (e.g., EKG, EEG). The probe takes advantage of this good high-frequency performance as follows: the electrode tip is mechanically moved back and forth at a frequency of more than 200 times per second between two positions in the medium. As the electrode moves up and down, between one voltage at point + and another voltage at point −, it “sees” a constantly varying potential (Fig 1, bottom). The tiny voltage drop (i.e., the potential gradient) that exists between the excursion limits of the tip is thus mechanically "transformed" into a high-frequency signal.

The motion of the probe is controlled by a piezoelectric element that is driven by a sine wave of known frequency. Because the electrode tip moves up and down at the same frequency, the phase and frequency of the sinusoidal signal measured at the tip is also known (Fig 1, bottom). Computational electronics can then be used to determine the magnitude of the tip signal with minimal interference from noise of other frequencies and phases. The voltage signal is also averaged over time.

Finally, in order to interpret the measurement as a current density, the local conductivity (in the region of the probe) must be known. Because the probe’s motion efficiently stirs the medium surrounding it, one can assume that the resistivity at the probe tip is the bulk resistivity of the medium. One can therefore multiply the solution’s bulk conductivity by the measured voltage gradient to obtain the current density (current per unit area) at the probe.

**Solutions** The saline solutions for these initial studies were chosen with several factors in mind. Electrode noise increases at low conductivities [11], and so these solutions represent a "worst-case" situation for probe use. As noted in Table I, solution resistivities, as measured with a conductivity meter (model 31A, YSI, Yellow Springs, OH), ranged from 26 Ω-cm (approximately that of sea water) to 82 Ω-cm. Whereas iontophoretic currents will usually be about two orders of magnitude greater than these estimated noise currents, this noise is of concern when attempting to detect endogenous or background currents.

Additional considerations were that these electrolyte solutions be physiologically compatible, that they represent the range of pH tolerated by the skin [4–8, see [20]], and that they be solutions that might reasonably be used in an iontophoretic experiment (see [6]). The pH 4 buffer consisted of 0.083 M Na₂HPO₄, 0.059 M citric acid, and 0.427 M NaCl. The pH 8 buffer was made from 0.195 M Na₂HPO₄, 0.0028 M citric acid, and 0.041 M NaCl. The components of the isotonic Sorensen phosphate buffer were 0.027 M Na₂HPO₄, 0.040 M NaH₂PO₄, and 0.079 M NaCl; the half-isotonic PBS was made using half these amounts. Except where noted, the same solution was used in the top and bottom of the chamber. All solutions were made in deionized water (R = 10–11 MΩ), with chemicals as received. The pH values at 25°C were measured with an electrode and meter (Model 250, Corning Glass, Corning, NY) calibrated with standard solutions of pH values of 4, 7, and 10 (Fisher, San Francisco, CA).

**Experimental Chamber** The skin samples were mounted in a modified Ussing chamber [21] made of lucite (Fig 2), with the epidermis exposed for microscopic examination and probe access. The disc of skin (cemented to the washer on the epidermal side) was supported from underneath (and kept flat) by a circular piece of nylon mesh with 125-μm openings (CMN-125, Small Parts, Miami, FL) set into the center of a second washer (Fig 2). Silicone grease (Dow-Corning, Midland, MI) was applied to the top of the washer to create a seal with the top half of the chamber. A seal was created with the lower half by a slight compression of the dermis when the two chamber halves were connected with nylon bolts. Hence, when the cell was assembled, the two sides of the skin were electrically isolated from each other. The lower half of the cell was closed with a circular glass coverslip, which allowed inspection for air bubbles after filling with saline.

The lower chamber could be perfused through two ports made from stainless steel needle tubing (#19, Fisher, San Francisco, CA). Perfusion of the top surface of the tissue was accomplished by
Table I. Solutions Used in the Experiments

<table>
<thead>
<tr>
<th>Solution</th>
<th>pH [25°C]</th>
<th>( \rho ) (Ω-cm)</th>
<th>( L_s ) (µA/cm²)</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH4</td>
<td>4.6</td>
<td>26</td>
<td>0.26</td>
<td>Citrate buffer</td>
</tr>
<tr>
<td>pH8</td>
<td>8.3</td>
<td>45</td>
<td>0.20</td>
<td>Citrate buffer</td>
</tr>
<tr>
<td>79 mM Sorenson</td>
<td>6.6</td>
<td>50</td>
<td>0.19</td>
<td>Half-isotonic PBS</td>
</tr>
<tr>
<td>0.158 M NaCl</td>
<td>4.8</td>
<td>78</td>
<td>0.15</td>
<td>Isotonic, unbuffered</td>
</tr>
<tr>
<td>158 mM Sorenson</td>
<td>6.5</td>
<td>82</td>
<td>0.14(6)</td>
<td>Isotonic PBS</td>
</tr>
</tbody>
</table>

*1 = estimated rms current noise of probe during clamping. The expected rms current noise for a current-clamped probe with an RC filter at the lock-in output is approximately 160/(\(|\langle I_d \rangle^2/\rangle|\) µA, where \( I_d \) = length of line of vibration, \( \rho \) (Ω-cm) = resistivity of medium, \( t \) (seconds) = time constant of lock-in, and \( D \) (µm) = diameter of probe tip [11].

For our experiments, typical values were \( l = 20 \), \( t = 2 \), and \( \rho \) as noted.

single inlet tube and suction (not shown), which maintained the pool of saline at a set level. Separate ports in the top and bottom halves of the chamber permitted KCl/agar bridges (3M KCl in 3% Difco agar), cast in polyethylene tubing (P.E. 160, Intramedic, Clay Adams, Parsippany, NJ) to be inserted to within 2 mm of the skin surface. The 15-cm-long bridges were inserted into Calomel reference electrodes (miniature glass body reference, Fisher, San Francisco, CA), which were used to measure the transdermal potential. The calomel electrodes were shorted together with a KCl/agar bridge (and both outputs grounded) when not in use. Another pair of ports allowed the insertion of a miniature thermocouple.

In the top and bottom compartments, circular Ag/AgCl wires (radius = 8 mm, 1.0-mm thick, 99.99%+, Aldrich, Milwaukee, WI) were used as current delivery electrodes when the current across the preparation was held constant. Preliminary scans with the probe demonstrated that this geometry provided a uniform current density at the surface of the skin for a distance of at least 5 mm from the center of the preparation, and so all measurements were made within this radius. The silver wires were rechloridized after each experiment using a standard protocol [22]. Two platinum black electrodes on the top surface of the chamber served as reference and ground for the vibrating probe. The coatings on the platinum wire (3.8 cm, 0.5 mm thick, Grade 1, Alfa, Danvers, MA) were inspected after each experiment, and renewed if necessary with the same procedure used to platinumize the probe electrodes (see below).

**Vibrating Probe: Methods** The probe vibration frequency was chosen so that the probe tip moved in a line along one axis. Vibration amplitude was then set to be approximately one probe diameter (15–20 µm in these experiments). Note that spatial resolution, which is a function of the size of the currents being measured and how close the probe can operate to the preparation, is usually comparable to the probe excursion used. Calibration of the probe signal (which is described in more detail below) was accomplished by measuring the electrode's response to a known current density [13].

To measure transdermal iontophoretic currents, the preparation was current-clamped (i.e., a set value of current was maintained across it), and the probe was vibrated along a line perpendicular to the plane of the skin, and near to its surface. Currents into the tissue were detected by scanning over its surface with the probe until a localized peak of current density was found (Fig 3). As current density is inversely proportional to the square of the tip-to-source distance, this distance had to be kept constant. In practice, this meant that the sample had to be as flat as possible, and the probe/issue distance checked often. Probe position was determined by first focusing on the surface of the preparation, then using the micro-

![Figure 2](image-url) Figure 2. An Ussing chamber for vibrating probe studies.

![Figure 3](image-url) Figure 3. The detection and localization of current into (and out of) the skin (top). Strong sources can mask weaker ones, particularly if the probe is too far away from the surface (bottom).
scope fine focus to raise the focal plane to the desired height above the tissue (usually 35–50 μm), and finally positioning the probe tip in this plane with a micromanipulator. The focal plane was returned to the skin surface, and loss of tissue flatness was thus detected when the skin surface moved out of the plane of focus.

These experiments were performed at the Vibrating Probe Facility, Marine Biological Laboratory, Woods Hole, MA, which is equipped with an automated data-acquisition system that computes and records the two-dimensional field measured (see Scheffey [23] for a description of this system). The measured current vectors can be superimposed on the video image of the experimental preparation (for example, see Fig 6 below). Motomicrometers are used to move the microscope (a modified Zeiss standard) along its Z axis, and to manipulate the probe electrode in X, Y, and Z. A trackball is used to navigate the probe in the area of interest. Video frames and probe data (time of measure, probe position, current vector magnitude and direction in X and Z, and comments) are stored on a hard disk, and can be redisplayed or printed out later for analysis. Time constants for both lock-in amplifiers were set to be between 0.1 and 10 seconds.

**Probe Fabrication and Setup**

Probes were fabricated from parafilm-coated stainless steel wires (length = 1.2 cm, diameter = 250 μm, SS300305, MicroProbe, Clarksburg, MD) that had had 5–7 μm of the insulation removed from their tips [23]. The tip of each electrode was gold-plated and platinumized [13,23] to a final tip diameter of 15–20 μm. Electrodes with a final tip capacitance of less than 8 nF were not used. Each probe was bent so that the wire made an angle of approximately 40° with its connector, so that the probe/vibrator assembly had sufficient clearance between the Ussing chamber and the microscope lens (Fig 2).

The probe was mounted on a two-dimensional vibrator consisting of three piezoelectric bimorphs (PZT-5H, Vernitron, Bedford, OH) in a Y-shaped linkage (Fig 4) that moved the probe in two roughly orthogonal directions, and thus permitted simultaneous measurement of the voltage gradients in the Z and X directions [23,24]. Separate pairs of lock-in amplifiers were used to measure probe output in each direction. The amplitude and linearity of probe motion along each axis was independently set by direct observation, with the video microscope looking down along the Z axis (and thus at motion in the X-Y plane), and a second monocular microscope looking along the Y-axis (to observe probe travel in the Z-X plane). Linearity meant that the probe tip was capable of moving in a line 3–4 probe diameters long at that frequency. When an appropriate driving frequency was located, the amplitude was adjusted so that the center-to-center excursion of the probe was one-tip diameter. The very small arc described by the tip motion was well-approximated by a line (i.e., the chord of the arc). The probe excursion in X is thus parallel to the skin surface, whereas the angle between the line of the tip motion along the Z-axis and the skin is 90°. As the skin is not perfectly flat at micron resolution, the probe is positioned 40 ± 10 μm above it, so small inhomogeneities may be ignored. Once the X and Z motions had been individually established, the probe was driven with both frequencies simultaneously and the two-dimensional tip motion was visually verified.

**Probe Calibration**

Calibration of the probe was performed in a small petri dish filled with the saline solution to be used in the experiment, with the electrode tip well below the surface, and approximately as deep as it would be over the tissue. A calibration current (I) was injected into the solution by a glass microelectrode (with the solution grounded using an Ag/AgCl electrode more than 1 cm away). The electric field near the microelectrode tip is radially symmetric (assuming that the drawn capillary is thin enough near the tip to ignore its blocking action on the field), thus the current density through a sphere of points at some distance r from the tip is constant (Fig 5). The probe can thus be calibrated by measuring the current density at a given radius r_m, where the current density at r_m is 1/4πr_m^2.

The probe was calibrated in the X-direction by bringing the tip of the probe and the tip of the current injection electrode into the same focal plane (lens used was a Zeiss UD40/0.65, 25.8 ×), and using an ocular micrometer (10:100, Carl Zeiss, Thornwood, NY) to position them 50 μm apart on the X-axis. The radial current was set to be 10 μA·cm^-2 at 50 μm, and was measured with the probe vibrating along the X-axis only. This determined the calibration constant for X-axis currents. Calibration for Z-axis currents was accomplished by repositioning the current injection electrode 50 μm above the tip of the probe and proceeding in a similar fashion. Figure 6 shows the X and Z calibration current vectors superimposed on the video image of the probe and the current injection electrode. Note that the horizontal vectors represent current flow in the plus and minus X directions, but that the vertical vectors repre-

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* The same sine frequency was used to drive both the base bimorphs (moving the probe tip back and forth along the X-axis), with a second (different) frequency simultaneously applied to the "springboard" bimorph (moving the tip up and down along the Z-axis). The frequency used to power the base bimorphs was chosen so that it was at least 200 Hz away from the frequency powering the "springboard" bimorph, so as to avoid cross-coupling between resonant modes. Harmonics of 60 Hz were avoided for the same reason. The frequencies used were between 200–800 Hz [24], with driving voltages of 2 V peak-to-peak.
Figure 6. Probe calibration. The long dark object on the right is the probe, and the thin lighter object coming from the bottom of the image to intersect it is the current injection electrode (compare with Fig 5). The white arrows are the current vectors; note that two such vectors are superimposed to make the double-headed arrows seen here. The horizontal vectors (measured earlier, when the probe was positioned at a distance $r$ to the right of the electrode tip) represent current flow in the $X$ direction, whereas the vertical vectors (measured with the probe positioned at a distance $r$ over the current injection electrode tip, as in this view) show ionic currents in the $Z$ direction (in and out of the plane of the picture). Scale bar, 10.0 $\mu$A/cm$^2$.

sent inward (down) and outward (up) current flow along the $Z$ axis. Figure 7 is a schematic diagram of the photograph in Fig 6.

Ussing Cell Setup Once the skin washer was mounted in the Ussing cell, the bottom chamber was filled with electrolyte and the two lower perfusion ports were joined with polyethylene tubing (P.E. 60, Intramedic, Clay Adams, Parsippany, NJ). The chamber was then attached to an X-Y-Z positioner (assembled from three translation stages, #420-05, Newport Corp., CA), and a pool of saline was deposited on the epidermal surface in the open top half of the chamber (with the perfusion port closed to prevent the saline from draining off). The chamber assembly was allowed to equilibrate to ambient temperature during probe setup (about 40 min). In order to approximate the clinical conditions of iontophoresis, the top of the chamber was not perfused during the experiment; however, the liquid level above the skin was carefully monitored. Attempts toperfuse the lower chamber were not successful, because small pressure changes caused the skin to rise off the supporting mesh. The lower chamber was consequently a closed volume for the duration of each experiment. The temperature of the cell saline (top and bottom), measured with a thermocouple before, after, and occasionally during an experiment, was $24 \pm 1^\circ C$. The top and bottom of the chamber each held 2 ml of liquid.

The ends of the agar bridges were trimmed with an oil-free razor blade before insertion into the chamber ports. Calomel offset (if any) was electronically zeroed with the two half-cells shorted together with an agar bridge. The transdermal potential was determined before the current clamp was applied, and at intervals during the experiment, as a measure of the integrity of the preparation. Potential measurements were made with the clamp off; at all other times, the calomel electrodes were open-circuited to prevent a shunt current path. The area of interest was scanned for endogenous currents prior to clamping and after each clamping experiment. The pH of the solution over the skin was measured before the clamp was first turned on, and after the final scan, with a miniature pH electrode (Model PHM-146 micro pH electrode, Lazar Scientific, Los Angeles, CA) and the pH meter.

Experimental Procedure The current densities used in these experiments (0.01–0.47 mA/cm$^2$) were in the range likely to be employed clinically [6,25]. The distribution of current above (45–50 $\mu$m) the surface was determined by moving the probe in a set pattern over the preparation and measuring the current vector at each point. Probe scans were restricted to a central area of the preparation where preliminary measurements had determined that the clamping field was uniform. When a point of interest was located, the clamp was turned off, and a scan pattern around this location that avoided obstructions (e.g., nearby hair stubs) was marked onto the video monitor. After probe zero† was verified, and probe height above the preparation re-established, the clamp was turned back on and the preparation scanned according to the pre-set pattern. The time per scan varied with the acquisition time per point (2 seconds for all our measurements), the number of points (8–25), and how far apart they were. A typical acquisition rate using the trackerball was 2–5 points per min, with most of the time spent moving between locations. Except where noted, the clamp was turned off when the scan was completed.

The video image and the probe output on the oscilloscope were monitored for probe contact artifact during the scan. The probe was checked for drift periodically by remeasuring electrical zero at 250–400 $\mu$m above the tissue surface (with the clamp off). Because of the possibility of current-induced damage to the skin, and concern about changes in upper medium toxicity, each experiment ended when a total of 10 mA-min/cm$^2$ of current had been delivered to the preparation (i.e., from 20 min at 0.47 mA/cm$^2$ to 1 h at 0.16 mA/cm$^2$), or when the liquid level of the open top of the cell had dropped more than 1.0 mm. Probe calibration was accurate to within 5% when remeasured after several runs.

RESULTS

Tests of Probe Operation To demonstrate that the probe could reproducibly vectorize currents, we measured currents injected into a dish of saline (as in the calibration procedure). Duplicate sets of measurements were made in 0.158 and 0.079 mM PBS. Currents of one-tenth to three times the calibration value of 10 $\mu$A/cm$^2$ produced vector magnitudes within 3% of the expected value, with vector directions varying by less than 1.2°. The low resistivity solution exhibited the greater variation in vector magnitude and direction. Similar results were obtained for currents in both the $X$ and $Z$ directions.

An additional control experiment was the scan of a Nucleapore polycarbonate filter (diameter 25 mm, pore size 5.0 $\mu$m, Nucleo-

† Drift of probe zero was checked periodically during the experiment (with the preparation open-circuited and the clamp off) by making a measurement far above the surface (250–500 $\mu$m). The probe tip waveform was monitored with an oscilloscope, as were the in-phase and quadrature (90° out of phase) outputs of the lock-in amplifier [12,17] so that artifactual measurements could be distinguished.
pore Corp., Pleasanton, CA) acting as an artificial membrane in the Ussing cell. The solution on both sides of the membrane was 158 mM PBS. With the clamp disconnected, very low noise currents ($\leq 0.1 \mu A$) were detected. When the clamp was connected, but not powered, the noise increased somewhat (0.2–0.5 $\mu A$), probably because the preparation itself was not electrically shielded. A very small clamping current was sufficient to produce large vectors over and near membrane pores. These vectors became progressively smaller as the probe was moved away from the pore.

The vectors measured by the probe did not change significantly (i.e., more than noted above) for measurements made 45 min apart. It did not matter if probe vibration was on or off between measurements, but noticeable drift of probe zero (up to 0.5 $\mu A$) could take place if current injection was maintained during the interval. In the course of the iontophoretic experiments, we found that current clamping at higher levels did eventually produce a small offset in the probe output. In consequence, probe reference was verified (and reset if necessary) after each scan. Another factor that could affect the measurement was the depth of the probe in the solution [17]. To avoid surface tension and other interface effects, the probe tip had to be at least 1 mm beneath the surface of the saline pool. Additionally, changing the immersed length of the probe more than 3–4 mm caused a shift in the output, probably because of increased vibrational damping.

**Superficial Anatomy of Mouse Skin** As seen under the microscope, light-colored bands of loosely clustered sebaceous glands alternate with darker bands of gland-free skin, giving the epidermis a somewhat striped appearance. Glands with an eruption of sebaceous material above them occasionally appear. As noted above, female hairless mice of this age have a few large (guard) hairs. The stubs of the clipped guard hairs appear to be quite long in the field of view. They had a small mound of tissue at their point of emergence from the skin, and the hairs could have visible follicular pits surrounding them. No relation between gland pattern and hair follicles was apparent.

**Background Scans** Background scans of each area (with the clamp off, and the calomel open-circuited) typically showed very small vectors with no particular orientation, which were due to a small amount ($\leq 0.5 \mu A$) of noise and offset in the electrode. A small ($\approx 3–10 \mu V$) passive potential exists across in-vitro mouse skin. This endogenous potential was large enough so that any damage (i.e., a microscopic hole created during the preparation of the sample) to the skin might be detectable as a leakage current. No such currents were found, even when the electrode was brought to within 20 $\mu m$ of the surface. Attempts to "amplify" flow through such "pores" by placing 0.079 mM PBS on the top of the skin, and 0.158 mM PBS in the lower chamber of the cell, did not elicit significant currents.

**Results of Current Clamping** Scans of the current-clamped skin detected two sorts of flows. The first consisted of large, localized inward or outward (Z) currents, up to several hundred microamperes, which were usually associated with visible structures (e.g., the hair in Figs 8 and 10). The flows were localized in the X-Y (skin surface) plane by scanning horizontally and looking for the point(s) with a large Z component and a small X component (i.e., a vector with "Y" large and X small, see Figs 3 and 11). Vertical (Y-axis) scans at the point of maximum current established source location along the Y-axis. Most interestingly, under certain conditions these localized flows could disappear (Figs 12 and 13). Occasionally, we also found small, non-localized (6–40 $\mu A$) inward or outward currents (Fig 14), usually in the vicinity of sebaceous glands (but sometimes occurring elsewhere). If large vectors, with a strong X component, were found over the entire field of view, a damaged, leaky, or poorly sealed preparation was indicated. Substantial vectors near the periphery of the observed area that did not correlate with an obvious structure usually indicated a sink or source outside the field of view. Very small currents of 1–3 $\mu A$, without structural association, and often lacking a consistent orientation, were also detected. These appeared to be due to a combination of clamp-associated offset, noise, and contributions from nearby sources.

Figures 8 and 10 show typical site-specific flows in the vicinity of a guard hair. These (and all subsequent) photographs are looking down at the surface of the mouse skin. Figure 9 is a schematic of Fig 8, included to aid in the interpretation of the low-contrast images. The light-colored vertical bar just to right of center in the picture is a guard hair shaft. The clusters of bright, circular areas to either side of it are sebaceous glands. The white arrows are the current vector superimposed on the video image. These indicate a strong source (the follicle), which was located during a scan with the transdermal current clamped at 0.063 mA/cm².

The experiment began with a check of probe electrical reference (with the clamp off) and its height (50 $\mu m$) above the preparation. A scan pattern around the source was then mapped out. In this instance, the hair in the center mechanically blocked the probe from measuring currents in the upper left quadrant of the frame. The clamp was then turned on, and the iontophoretic currents were measured. The solutions used were isotonic PBS (bottom), and half-isotonic PBS (top).

Figure 8 shows ionic flow in the vicinity of the hair follicle.

![Figure 8. Currents near a hair. The hair is the bright vertical bar in the center, and the white circular objects are sebaceous glands. Note the pronounced inward flow (downward arrows) at the base of the hair. Scale bar, 100 $\mu A$/cm².](image1)

![Figure 9. Identification of features in Fig 8. The vectors are black in the diagram and white in the photograph.](image2)
Recall that whereas the horizontal component of each vector represents current flow in the X direction, the vertical component represents inward (down) and outward (up) current flow along the Z axis. The large vectors in the center of the image indicate that current is flowing into the skin near the base of the hair. For low current densities and buffered preparations, vector size was a function of clamp level; that is, increasing the clamp strength increased the magnitude (length) of each vector without substantially changing its direction. The three vectors on the right side of the picture, as well as the two on the left edge, show currents flowing predominantly over the skin (in the X-direction) rather than into it (as indicated by vectors in the Y-direction). The total scan time was 4.5 min, and the initial transdermal potential difference was 3.9 mV. Scale = 100 μA/cm².

Figures 8 and 10 also illustrate the reproducibility of probe measurements in a stable preparation. The measurements in Fig 10 were made using the same scan pattern as in Fig 8. The first reading was taken with the clamp on (arrowheads with shafts), and the second with the clamp off (arrowheads without shafts). These vectors are all from the same scan, therefore, rather than scans superimposed. Comparison with the previous photograph demonstrates that the current detected in each case are nearly identical, and are thus not artifacts of the measurement procedure, i.e., that the measurements are reproducible. Total scan time was 8.0 minutes. The period between the scan in Fig 8 and these measurements (4.5 min) included a “clamp off” scan, as well as a check of probe reference and its height above the tissue. Note that the “clamp off” scan was very similar to the initial background scan. The stable potential difference, measured after the clamp had been turned off for several minutes, was 6.25 mV. Scale = 100 μA/cm².

As a rule, strong flows occurred at specific sites, particularly hairs and what appeared to be extrusions of sebaceous material. One such extrusion is shown in Fig 11, which shows currents in the vicinity of a slightly swollen sebaceous gland. This scan also shows (a) how flows were localized in X and Z, and (b) the spatial resolution of the probe, with the strong outward currents falling off rapidly as the electrode moves away from the source. The skin, in 158 mM NaCl, was clamped at 0.135 mA/cm². The pH of the top solution changed over the course of the experiment (which included measurements in addition to those shown) from 4.73 to 5.5, and the transdermal potential difference dropped from an initial value (before the clamp was applied) of 7.4 mV, to a stable potential difference of less than 3.4 mV. Scan time was 11.5 min, and the electrode was 45 μm above the tissue. Scale = 100 mA/cm².

Small guard hairs were more likely to be sites of flow than large guard hairs, but it was not possible to predict by inspection if a particular hair would have current flowing through its follicle; many hairs did not. Sebaceous extrusions always almost showed some evidence of ionic flow near their location. Large localized flows that did not appear to be associated with any particular feature of the skin (or may have been masked by its translucence) were occasionally detected as well. Figure 12 shows one such source, which had disappeared by the time the area was re-measured 50 min later (Fig 13). Such dramatic changes as this disappearance were unusual, but relatively rapid changes in vector magnitude and direction were not uncommon in unbuffered preparations (such as this one), and in preparations subjected to high current densities. This sample was clamped at 0.153 mA/cm² and scanned at 50 μm (the solution was 158 mM NaCl); scale, 50 μA/cm². Whereas changes in vector magnitudes were observed at all buffered pHs, the rate of change was far slower than with the unbuffered preparations. Vector direction changed as well in the pH-8 samples, but did not in the other buffered preparations.

Small, non-localized flows also occurred in all preparations, but appeared more frequently in the pH-8 samples, as illustrated in Fig 14. These currents were detected by an electrode 50 μm above the tissue, in a 10.25-min scan with the skin clamped at 0.015 mA/cm². Whereas there are several sebaceous glands and a small hair in the vicinity, the flow observed did not appear to be associated with them; scale, 10 μA/cm². Whereas the low-clamp current density makes comparison with the other photographs difficult, these data do show that currents elicited by such low densities are detectable. The pH-8 samples also had a roughened appearance, which was due to a much larger number of superficial sebaceous extrusions than were seen in any other preparation.

**DISCUSSION**

Given the evidence in support of iontophoretic transport via pores [3–7], the existence of site-specific flows was not surprising. The currents detected are, however, direct evidence that skin appendages act as channels for the flow of ions through the skin (“pore” localization in the dye studies might, for example, have been due to the dye binding at sites distinct from pores). Whereas similar evidence has been obtained with pulsed currents and scanning microelectrodes in combination with iontophoresed dyes [7], the vibrat-
ing probe electrode measures flow as it is taking place, rather than characterizing the pore after iontophoresis.

The micropipette scanning technique has other flaws, in that (a) the high drift and thermal noise associated with saline-filled electrodes make them difficult to use for low-frequency measurements; (b) the pulses used to make the measurements may themselves cause changes in the skin; (c) the assumption that the solution resistivity at any point is the same as the bulk resistivity is not a good one, as the volume is not well-stirred; and (d) the relatively large electrode separation necessary for a reasonable signal-to-noise ratio limits the spatial resolution of this arrangement, because other currents in the vicinity contribute strongly to the measurement.

The vibrating probe method differs from pore location by dye iontophoresis in that (a) it has better spatial resolution (5–40 μm versus 2 mm), which aids in structure-flow correlation; (b) it has far greater temporal resolution (on the order of seconds, as opposed to 30 min [7]), and so can monitor changes in flow resistances over short periods of time; and (c) it can detect sites with a wide range of resistances (whereas a dye appears to require a time-invariant low resistance channel).† Whereas we have demonstrated pathway localization for small ions only, there is no a priori reason that the probe should not be sensitive to the ionic flows of species with larger molecular weights (e.g., charged peptides). We thus expect to be able to directly measure the flow of an iontophoresed drug as opposed to a dye model. Lipophilicity, size, and steric considerations are important factors in transdermal transport, and must be carefully controlled in experiments designed to ask mechanistic questions. The vectorization of flow by the two-dimensional probe provides a more complete picture of charge transport in the vicinity of a source or sink, and the ability to visualize both X and Z current aids in both the location of “invisible” sources and the detection of damage or leaks in the preparation. The current levels used in iontophoretic experiments are such that the signal-to-noise ratio is very high, and the use of a very short time constant (i.e., data acquisition time for the probe) is practicable.

Our results show that large iontophoretic currents tend to flow at what are presumably low-resistance particular sites, although the microstructural detail of these sites is not yet known. Other investigators have found both follicular and non-follicular pathways [5,7], and one might speculate that low-resistance sites are places where the epidermal barrier layer is thin (e.g., at the base of the hair follicle), or disrupted (as might be the case for the sebaceous extrusions). The range of follicle-current carrying capacity (from no current to large vectors) may be an indication of the metabolic state of the hair. Active (growing) follicles could disturb the barrier and create leakage paths, whereas quiescent (or encysted) follicles would be surrounded by an effective seal. The small, non-localized flows that appeared primarily in the pH-8–treated skin may have been due to weakening of the barrier in these samples, whose roughened appearance suggested that tissue alteration had in fact taken place.

This finding—that most electrically facilitated transport takes place at particular sites in the skin—has potential clinical significance as well. It has been recognized that such “focusing” of current

† The pore density detected by Burnett [7] was much smaller than the number of shunt paths in the area measured (at least 300/cm², including eccrine sweat glands and hair follicles, see [26]). This suggests that the fluorescein travelled primarily along the paths of least resistance, and, furthermore, that the conductivity of the detected paths did not significantly decrease (and thus reduce or stop the flow) during the course of the experiment.
flow may lead to cellular damage from a variety of mechanisms, such as Joule heating [2]. In addition, iontophoretically transported drugs will be effectively concentrated at these surface sites, and probably in the pathways taken through the skin as well. As a result, cells along the transport paths will be exposed to much higher drug concentrations than cells in non-current-carrying areas. Irritation and allergic reaction may thus be seen at rather low surface concentrations.

The tendency of the current vectors in a buffered, and/or low-current preparation (such as in Figs 8 and 10) to maintain their magnitude and direction, while currents in unbuffered (and high-current density) samples did not (Figs 12 and 13) is consistent with the results of diffusion-cell experiments performed in our laboratory [27]. The iontophoretic transdermal delivery of morphine sulfate reached a "steady-state" value if the donor solution was buffered at either pH 5 or pH 7; on the other hand, if no buffering was used, the pH in the donor phase changed dramatically, leading to a catastrophic degeneration in the barrier and a steep increase in drug flux. Other investigators have reported similar results [28].

Whereas it is convenient to think of ionic flow through pores as the movement of charge through a parallel network of electrical resistances, there are certain caveats to this model. (a) Different solutes flowing through the same pore may experience different resistances [6], (b) Pore resistance varies with time, and is affected by solution pH and imposed current density. The rate of change of vector magnitude was much greater in unbuffered preparations and for higher current densities, but some change occurred in all cases. Note, however, that a change in vector magnitude and direction at a particular point (with the clamp constant) could be caused by either conductivity change at the site of flow or a change in the conductivity of a neighboring site. (c) In constant-current iontophoresis, the bulk current through the preparation is maintained at a set level by adjusting the voltage across it. The resistance of an individual pore may be constant or vary with time, but in either case changes in transdermal voltage will affect the amount of current passing through it. In other words, steady-state bulk transport does not imply that steady-state conditions exist for each current-passing element.

A related question is that of simple viability. Measurement of the potential did not provide useful information about the state of the tissue, although the capacitive effects observed did suggest an explanation for the Z reversals seen at high current densities (see below). Viability may be a factor in iontophoretic transport if, for example, a large fraction of transport takes place through live tissue (such as the base of the follicle), or if transport through non-viable pathways is affected by current-induced changes in living cells [19].

The current density measured by the probe over a given source will be contaminated to some extent by currents from nearby sources. For a measurement at a height of h directly over a source, the contribution to the signal from a second source, of the same size, at a distance d from the first, is \( h^3/d^3 \) [13,17]. In the case of mouse skin, the current sources tended to be sparsely distributed over an insulating surface (\( \approx 500 \mu m \) apart); some, however, were close enough to interfere with each other, and these were not evaluated. Lateral contributions can to some extent be reduced by bringing the probe closer to the skin surface, but this increases the chance of probe damage. A close approach can also "magnify" any effects due to the non-uniformity of the surface, because the angle of incidence of the probe excision to the plane of the source may change (e.g., as the probe gets nearer to a "bump" in the surface). Monitoring the probe-tissue distance is clearly of critical importance. One benefit of this requirement is that an exact knowledge of probe height (corrected for the refractive index of the medium, if an air/water interface exists) is necessary for quantitative measures of current. Such measurements, however, cannot be made as rapidly as the more qualitative scans performed higher above the surface.

Apart from the necessity for careful height control, which will make the in vivo use of the probe very challenging (primarily because of the need to keep the surface flat), the disadvantages and limitations of the vibrating probe, with respect to mechanistic stud-

ies of iontophoresis, are that: (a) it cannot directly measure the electro-osmotic flow of uncharged species in the direction of positive ion movement [6]; (b) it is non-selective, in that it is sensitive to all ionic flows occurring in the system; (c) it works best in high resistivity media (because the probe voltage signal is larger and the probe electrical noise is reduced), and thus measurements in solutions with resistivities less than \( \approx 25 \Omega \cdot cm \) will be quite noisy; (d) probe measurements at very high current densities can be difficult to interpret; and (e) it is technically complex.

**SUMMARY**

The vibrating probe can be used to locate and quantify sources and sinks of iontophoretic current in the skin. Whereas the procedure is technically demanding, probe measurements are reproducible within and between preparations, and can be performed in ranges of toxicity, pH, and current density useful to the mechanistic study of iontophoresis. The spatial (5–40 \( \mu m \)) and temporal (0.1–10 second) resolutions of the technique are sufficient for the acquisition of useful data. No information, however, is provided about the pathways taken inside the skin, and charge carriers are not identified by this technique.

On the basis of these studies, iontophoretic currents are primarily appendageal, and certain appendages carry more current than others. The magnitude and direction of these appendageal currents can change under some conditions, such as a high current density. It should be possible to make real-time measurements of the effect that changes in pH, toxicity, and iontophoretic current density or potential have on ionic flows, and to detect preferred routes for particular substances, if such exist.

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**REFERENCES**