Microelectrode Studies of the Active Na Transport Pathway of Frog Skin

S. I. HELMAN and R. S. FISHER

From the Department of Physiology and Biophysics, University of Illinois, Urbana, Illinois 61801

ABSTRACT When the outer surface of short-circuited frog skin was penetrated with microelectrodes, stable negative potentials that averaged near −100 mV were recorded consistently, confirming the results of Nagel (W. Nagel. 1975. Abstracts of the 5th International Biophysics Congress, Copenhagen. P-147.). The appearance of these stable potentials, V_0, was concurrent with the observations that (*a*) a high resistance outer barrier R_0 accounting for approximately 75% or more of the transcellular resistance of control skins had been penetrated and that (*b*) 10^{-5} M amiloride and reduced [Na] outside caused the values of V_0 to increase towards mean values near −130 mV while the values of $\%R_0$ increased to >90%. It was of interest to observe that the values of E_1 observed in studies of the current-voltage relationships were the same as the values of E_1' defined as the values of voltage at the inner barrier when the V_0 of the outer barrier was reduced to zero by voltage clamping of the skins. Accordingly, these data are interpreted to mean that the values of E_1, ~130 mV, represent the E_{Na} of the sodium pump at the inner barrier. 2,4-DNP was observed to decrease the values of V_0 to low negative values in approximately 10–15 min. For all values of transepithelial voltage $<E_1$ the V_0 was negative. These data can be interpreted with a simple electrical equivalent circuit of the active sodium transport pathway of the frog skin that includes the idea that the outer membrane behaves as an electrical rectifier for ion transport.

INTRODUCTION

The isolated frog skin bathed symmetrically with identical solutions is capable of generating and maintaining a transepithelial voltage in excess of 100 mV. The origin of this voltage has been attributed to the active transport of sodium (Ussing and Zerahn, 1951) and, according to the hypothesis advanced by Koefoed-Johnsen and Ussing, the transepithelial voltage was ascribed to the sum of two series diffusion potentials originating at the outer (sodium electrode) and inner (K electrode) borders of the epithelial cells (Koefoed-Johnsen and Ussing, 1958). In part, support for this model was available from the microelectrode studies of Engbaek and Hoshiko where the voltage profile of the skin in most cases conformed to a two-step positive voltage increase as the microelectrode was advanced from the outer to the inner surface of the skin (Engbaek and Hoshiko, 1957). This finding was subsequently confirmed by others (Ussing and Windhager, 1964; Whittembury, 1964; Cereijido and Curran, 1965; Morel and Bastide, 1965; Biber and Curran, 1970; Rawlins et al., 1970) but challenged by Chowdhury and Snell (1965, 1966).

In the past several years, evidence has accumulated to support the idea that the cells of the stratum granulosum that constitute the first living layer of cells just beneath the stratum corneum may play a significant role in the process of active transepithelial sodium transport (Voûte and Ussing, 1968; Dörge et al., 1974). As these cells are only 5–10 μm in depth, attempts to penetrate them from the outer surface could conceivably be difficult and, despite the fact that small negative unstable potentials (outside grounded, skin open-circuited) have been observed upon initial penetration of the skin with a microelectrode, they have not been stressed in favor of the observed positive voltage steps consistent with the model proposed by Koefoed-Johnsen and Ussing. In this regard, it is of special interest to note the recent studies of Nagel who observed that microelectrode penetrations of the skin of *Rana temporaria* from the outer surface into cells of the stratum granulosum yielded stable intracellular potentials markedly negative (−90 to −100 mV) with respect to the outer solution even when the skins were open circuited (Nagel, 1975). Nagel also found that positive steps of voltage were not observed until the microelectrode was advanced into the deeper layers of the skin, presumably into the cell layers of the stratum spinosum and stratum germinativum. Moreover, it was observed that the magnitude of the negative potentials was increased by amiloride and changes of the sodium concentration at the outer surface of the skin. As these observations were entirely consistent with the view that the microelectrode had penetrated the sodium-sensitive outer barrier of the skin, it appeared likely that the cells of the stratum granulosum participated in the process of active sodium transport and not the cells of the stratum germinativum alone as suggested by MacRobbie and Ussing (1961).

The present studies were done to confirm and extend the findings of Nagel especially with regard to the interest of this laboratory in defining the location and magnitude of the E_{Na} of the sodium pump. As will be shown, our observations are remarkably similar to those of Nagel. Moreover, it was possible to demonstrate that the values of E_{Na} for transepithelial sodium transport are identical to the voltage developed across the inner barrier when the voltage (and presumably active sodium transport) across the outer barrier was reduced to zero. It was also possible to demonstrate that 2,4-DNP caused the values of E_{Na} to fall dramatically, thus providing evidence of its metabolic dependence. An electrical model is proposed that accounts for the electrical rectifying properties of the skin as disclosed by the I-V relationships of the entire skin and the I-V relationships of outer and inner barriers.

MATERIALS AND METHODS

Studies were done with abdominal skin of southern frogs (*R. pipiens berliendieri*; Nasco, Oshkosh, Wis.) bathed symmetrically with a chloride-Ringer solution containing 100 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 2.4 mM $NaHCO_3$, and 11.1 mM dextrose, aerated and bubbled with 100% oxygen.

Symbols

V_T, Transepithelial voltage, inside to outside with outer solution ≡ 0 mV;
V_{oc}, spontaneous value of V_T, open circuit;

V_o, voltage across outer barrier, cell interior with reference to the outer solution;
V_i, voltage across inner barrier, inner solution with reference to the cell interior;
E_1,E_2, voltages at "breaks" observed in the I-V plots of entire skin (see Figs. 7–9);
E_1', voltage across inner barrier when V_o = 0 mV;
E_{Na}, electromotive force of the sodium pump defined by Ussing and Zerahn (1951);
I_T, transepithelial current through entire skin at voltage V_T. (Positive currents are from inner to outer solution that cause hyperpolarization of V_T.);
I_s, transepithelial current through the shunt pathway = V_T/R_s;
$I_T^{E_1}$, transcellular current through the E_1 pathway of active Na transport = $(I_T - I_s)$;
I_1, transepithelial current when $V_T = E_1$;
I_{sc}, short-circuit current;
R_1,R_2,R_3, slope resistances, $\Delta V_T/\Delta I_T$ (see Figs. 7–9);
R_o^f, resistance of outer membrane, $\Delta V_o/\Delta I_T^{E_1}$, when V_o is negative;
R_o^b, resistance of outer membrane when V_o is positive;
R_i, resistance of inner membrane, $\Delta V_i/\Delta I_T^{E_1}$ (see text for other methods of estimation);
R_s, resistance of the shunt pathway = E_1/I_1;
R_{Na}, transcellular resistance to sodium current = E_{Na}/I_{sc} = $(R_o^f + R_i)$;
$\%R_o$, See Materials and Methods.

Skin Chamber Arrangement

The skins were mounted horizontally in a chamber shown diagramatically in Fig. 1. The chamber was machined from lucite and permitted the skins to be glued with the tissue adhesive Zipbond (Tescom Corp., Minneapolis, Minn.) between gaskets (19 mm OD, 9.5 mm ID, 3 mm thickness; area of skin, 0.72 cm^2). This permitted the skins to be mounted without edge damage as described previously (Helman and Miller, 1971). The lower gasket was sealed into a depression in the chamber with liquid Sylgard 184 and the inner surface of the skin was glued to its upper face. Consequently, the outer surface of the skin was oriented upward and accessible to penetration with the microelectrode from above. The second gasket was glued to the outer surface and this gasket formed an open well that contained Ringer solution through which the microelectrode was advanced perpendicularly towards the skin and that also permitted electrical connections to be made to the recording devices. No special grids or supports were used to prevent movement of the skin in the vertical direction.

Electrical Connections

Electrical connections were made to the inner and outer solutions with polyethylene bridges containing Ringer solution in 3% agar. The bridges also made contact with wells containing 1 M NaCl and Ag·AgCl electrodes which in turn were connected to the electrical apparatus. As shown in Fig. 1, a four-electrode system was used to measure the voltage across the skin, V_T, and to allow the transepithelial flow of current, I_T, in response to command signals generated at the voltage clamp. A simplified schematic representation of the circuit is shown in Fig. 2.

Amplifiers nos. 2 and 3 were Burr-Brown instrumentation amplifiers (model 3621, Burr-Brown Research Corp., Tucson, Ariz.) with differential input and impedance of 10^{13} Ω. Amplifier no. 2 was used to measure the voltage across the skin, V_T, and amplifier no. 3 recorded the voltage between the outer solution and the tip of the microelectrode, V_o. A voltage reference source was used to null the asymmetry in the microelectrode and other junction potentials. In practice, once nulled, the offsets remained within ±1 mV

even after many cells were punctured. In order to vary the voltage across the skin, voltage clamping was achieved with amplifier no. 1 providing positive feedback (Analog Devices 520 wired as a differential adder subtractor with gain of 10,000, Analog Devices, Inc., Norwood, Mass.) to the skin. The transepithelial current through the skin, I_T, was monitored as the voltage across a 1K precision resistor. Adjustment of the precalibrated hold command voltage permitted the skins to be clamped at any voltage and in the present studies the skins were short-circuited continuously except where indicated in the text. The voltage across the skin could also be changed in increments of 20 mV for intervals of 600 ms by triggering of the pulse command. As the current responses to step changes in voltage reach steady-state values well within this time interval, the steady-state values of V_T and I_T at voltages between −40 mV and +200 mV could be used to determine

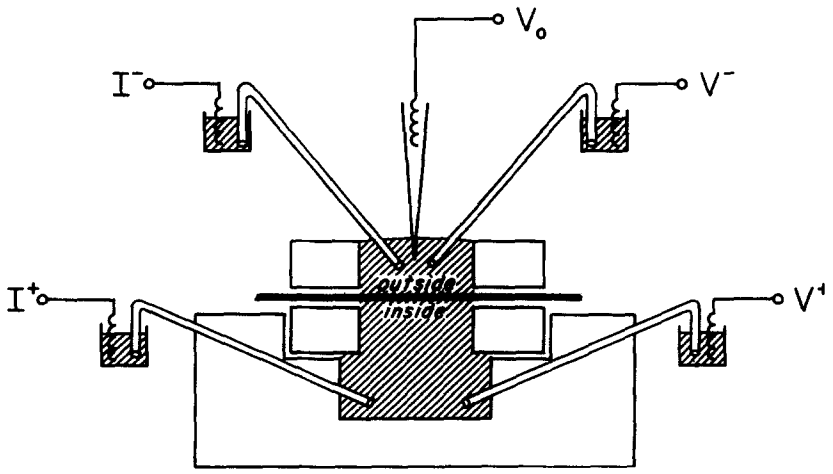


FIGURE 1. Skins were glued between circular lucite gaskets with the tissue adhesive Zipbond. Polyethylene bridges containing Ringer in 3% agar made contact with the inner and outer solutions and with 1 M NaCl in the wells. Ag·AgCl wires completed the circuits to the electrical apparatus (see Fig. 2). The voltage across the skin was measured as V^+-V^-, and transepithelial current flow occurred between I^+ and I^-. Not shown are inlet and outlet holes for changing inside solution.

the I-V relationships as before (Helman and Miller, 1971; Helman and Miller, 1973; Helman et al., 1975; O'Neil and Helman, 1976). All signals were fed to Gould Brush strip chart recorders (Gould, Inc., Instrument Systems Div., Cleveland, Ohio) for continuous monitoring of their values and/or to digital panel meters (Analog Devices, AD 2010) that displayed the digitalized values of voltage and current to 0.1 mV and 0.1 μA. Sample-hold modules built into the equipment also permitted the steady-state values of voltage and current to be read at approximately 580 ms after onset of a pulse command.

Preparation of Microelectrodes

Microelectrodes were pulled immediately before use on a horizontal puller (Narishige, Labtron Scientific Corp., Farmingdale, N.Y.) with microfiber glass capillaries (Frederick Haer & Co., Ann Arbor, Mich., 30-30-1). The tips were filled by capillarity (for approximately 2 min) by immersion of their barrels into 3 M KCl and the barrels were then back-

filled with 3 M KCl.[1] An Ag·AgCl electrode was inserted into the barrel and made contact with the electrical apparatus. Tip resistances varied between 20 and 40 mΩ and remained stable provided the tips were not forced deeply into the skin. No special precautions or techniques were found necessary to obtain tips adequate for these studies. In

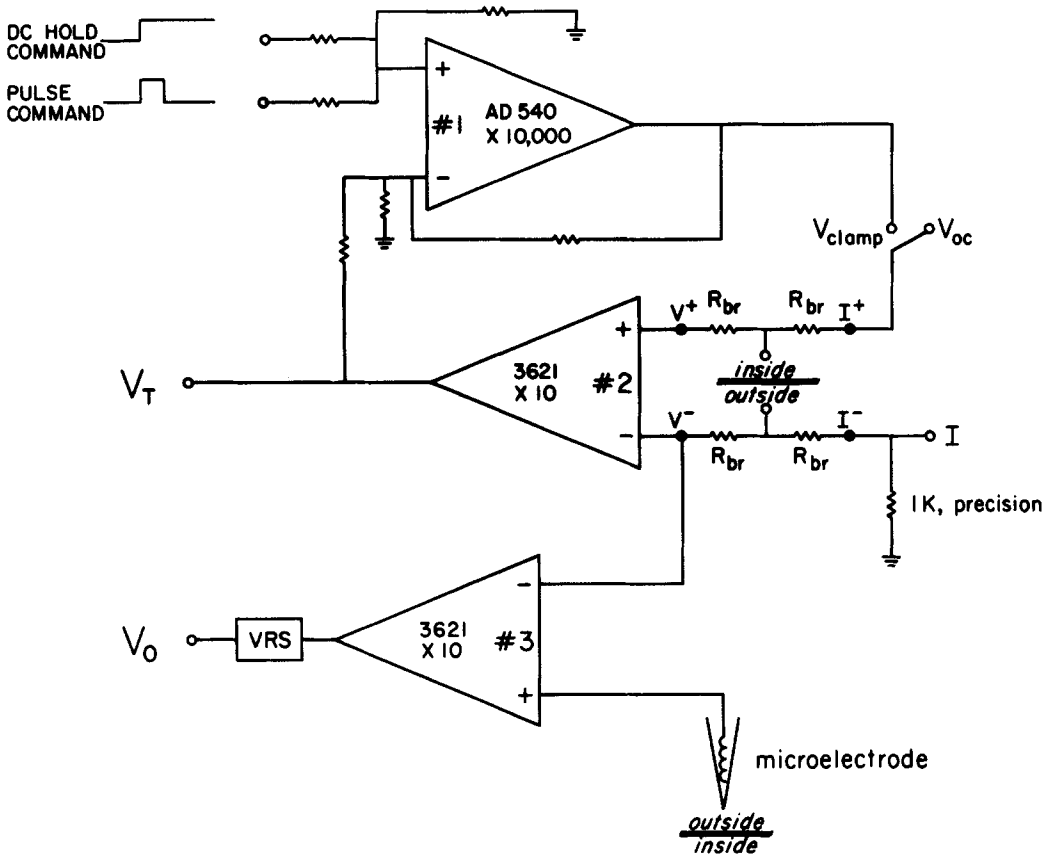


FIGURE 2. Electrical apparatus for voltage clamping of the skin and recording of microelectrode potentials (see text for details). The voltage reference source (VRS) was used to null asymmetries and junction potentials. The resistances of the polyethylene-Ringer bridges are labeled R_{br}. The DC hold command was set at 0 mV to maintain the skins short circuited. Pulses of variable voltage (600-ms duration) were used to clamp the skin at voltages between −40 mV and +200 mV. As reported previously (Helman and Miller, 1971), a pulse duration of 600 ms permits observation of the steady-state values of V and I.

some cases the tips were sharpened on a Brown-type electrode sharpener (David Kopf Instruments, Tujunga, Calif.) and as the results were the same, this procedure was not pursued.

[1] This method was especially useful, as it permitted the electrodes to be fabricated immediately before use, thus avoiding the etching of the tip that is usually associated with other methods of filling that require many hours. Electrodes used immediately or several hours after fabrication gave identical results.

The microelectrodes were mounted in a holder attached to a model MM-33 Eric Sobotka Co. (Sarasota, Fla.) manipulator that allowed the microelectrodes to be advanced in a direction perpendicular to that of the surface of the skin. This simple arrangement was found to be entirely adequate as evidenced by the ease of penetration of the skin and the observations of large stable intracellular voltages for prolonged periods of study (see Results). The entire apparatus was housed in a Faraday cage to minimize electrical noise (<100 μV). As an added convenience the voltage at the microelectrode was monitored with a voltage-controlled oscillator and fed to an audio amplifier.

Determination of R_o/R_i

In order to determine tip localization it has been customary to determine the relative resistance of the outer barrier (R_o) and the inner barrier (R_i) when the microelectrode is recording a stable intracellular potential. Such measurements are done routinely by passing a pulse of current through the epithelium and recording the changes in voltage at the microelectrode, ΔV_o, and across the entire epithelium, ΔV_T. Accordingly, the resistance of the outer barrier, expressed as a fraction or percent of transcellular resistance, can be calculated.

$$\%R_o = [R_o/(R_o + R_i)] \times 100 = (\Delta V_o/\Delta V_T) \times 100. \quad (1)$$

In the present studies, a voltage clamp was used to vary the V_T. Consequently, in order to determine the $\%R_o$, it was necessary to measure only the V_o at the command values of V_T. From a plot of V_o vs. V_T, the slope of the line gave estimates of $\%R_o$ (see Fig. 4 and Results). Although the present studies could have been done equally well with pulses of constant current, we believe it advantageous to use a voltage clamp as this permits the electrical dissociation of the paths monitored by the microelectrode from other parallel transport pathways whether they be active or passive in nature. More importantly in the present studies, the voltage clamp permitted us to obtain the current-voltage relationships as done before (Helman and Miller, 1971; Helman and Miller, 1973; Helman et al., 1975; O'Neil and Helman, 1976) from which the values of E_1 could be estimated in the same skins as used for study with the microelectrode (see Figs. 7–9).

In order to determine the specific resistances of the outer and inner barriers two methods were used that gave similar values of R_o, R_i (see Results)). In method A we assumed in accord with Ussing and Zerahn (1951) that the resistance to sodium flow R_{Na} could be estimated from the quotient E_{Na}/I_{sc}.[2] Accordingly, the specific resistances of the outer and inner barriers were calculated with the following equations:

$$R_o = (\%R_o/100) \cdot R_{Na}, \quad (2)$$

or

$$R_o = (\%R_o/100) \cdot (E_{Na}/I_{sc}), \quad (3)$$

and

$$R_i = R_{Na} - R_o. \quad (4)$$

In the present studies the values of E_{Na} were estimated from either the values of E_1 (I-V relationships) or, equally well, the values of E_1' (microelectrode studies, see Results). As

[2] R_{Na} calculated in this way presumes that net sodium transport occurs via a single active transport pathway characterized in part by its E_{Na}. However, data are available that support the idea that active sodium transport in the skin occurs via two parallel active pathways (Helman, 1975; see footnote 3; Macchia and Helman, 1976) with 90–95% of the sodium current carried via the E_1 pathway. Accordingly, the short-circuit current I_{sc} to be used to calculate R_{Na} of the E_1 pathway is some 5–10% less than the I_{sc}, and thus the R_{Na} estimated from the values of I_{sc} will be underestimated by this amount or approximately 5–10%.

the microelectrodes were undoubtedly recording intracellular potentials, it would be reasonable to ascribe the values of R_{Na} to a cellular pathway for sodium transport and not to an extracellular pathway, as has been suggested by Cereijido and Rotunno (1968). In accord with the views of others, active sodium transport proceeds via a cellular route with sodium entry occurring at the outer barrier and active sodium extrusion into the inner solution occurring at the inner barrier.

A second approach (method B) was used to estimate the specific resistances of outer and inner barriers. If the shunt resistance R_s is known, then the R_{Na} can be calculated with the additional measurement of the resistance of the skin R_{skin}, as done before (O'Neil and Helman, 1976), and subsequently the R_o and R_i calculated with Eq. (3) and (4):

$$R_{Na} = (R_{skin} \cdot R_s)/(R_s - R_{skin}). \quad (5)$$

In the present studies R_{skin} was determined directly from the slope resistances (R_2) of the I-V relationships (see Figs. 7–9). To estimate R_s, we relied on our previous observations

TABLE I

CHARACTERISTICS OF SKINS: CONTROL VALUES

Skin no.	V_o	I_{sc}, Mean	(Range)	V_{oc}, Mean	(Range)
	mV		*μA/cm²*		*mV*
1	−103.1±1.2 (12)	53.1	(49.0–57.2)	73.7	(50.7–96.7)
2	−98.4±1.1 (9)	75.6	(62.0–89.1)	90.0	(81.4–98.7)
3	−77.1±2.0 (30)	77.5	(54.6–100.4)	74.0	(68.6–79.4)
4	−77.4±1.1 (9)	11.6	(2.8–20.4)	47.5	(20.0–75.0)
5	−104.7±2.3 (10)	32.0	(24.4–39.7)	63.0	(50.0–75.9)
6	−100.6±1.6 (5)	6.8	(4.7–8.8)	44.0	(38.0–50.0)
7	−102.6±2.7 (12)	23.1	(16.9–29.2)	40.5	(38.0–43.0)
8	−113.7±1.5 (15)	24.9	(22.2–27.6)	34.5	(30.0–39.0)
9	−101.0±2.0 (2)	29.5	(25.4–33.6)	47.0	(44.0–50.0)
Mean±SE (9)	−97.6±4.1		37.1±8.6		57.1±6.3

Values of V_o are mean ± SE (number of cells). Most cells were studied for approximately 5–10 min. Values of I_{sc} and open-circuit voltage V_{oc} are mean values (range) over the entire experimental period of approximately 2.5–3 h.

that the quotient E_1/I_1 estimated at the coordinate values where the slope resistances R_1 and R_2 intersect gives values of the shunt resistance (Macchia and Helman, 1974; O'Neil and Helman, 1976). As will be shown (see Results), methods A and B yielded similar values for R_{Na}, R_i, and R_o despite the differences of approach and the assumptions required to obtain these estimates.

RESULTS

The abdominal skins of 12 frogs were subjected to study with the microelectrode after being allowed to remain short-circuited for approximately 30–60 min. Of these skins, nine (see Table 1) could be penetrated easily and repeatedly and were used for collection of the data. In the remaining three skins, penetration was difficult and we could not obtain penetrations that gave long-term stable values (>5 min). Nevertheless, the few recordings obtained for each skin gave values similar to those observed in other skins. In part, the difficulty in penetration of these three skins could be related to either the thickness or the tenacity of the stratum corneum and could depend on the phase of the molting cycle at the

time of study. Indeed, the difficulties encountered could not be attributed to differences between microelectrodes or other technical problems. Characteristically, appearance of the tip of the microelectrode at the stratum corneum was signalled by small voltages and the presence of noise. As the tip was advanced slowly, occasionally with a light tap of the manipulator, the noise would disappear with an abrupt change in V_0 that would remain stable to within 5 mV for up to 1 h or more. In some cases the tips were advanced into deeper layers of the skin and in every case the values of V_0 would fall to some more or less stable negative value but one significantly less than that observed initially. All attempts in all skins to advance the tips into deeper layers resulted in either breakage of the tip or a permanent shift in tip potential and resistance. We did not observe steps of positive potential while the skins were open circuited and none were expected while the skins were short circuited.

Typical records are shown in Fig. 3. The skins were short circuited and maintained so with the voltage clamp. Although not shown here, the short-circuit currents, I_{sc}, remained constant during impalement of the skin. In the two examples shown, the V_0 was stable at -102 mV for the control skin and -127 mV for the skin exposed to 10^{-5} M amiloride outside. In order to determine the relative resistance of the inner and outer barriers, the skins were voltage clamped at values of V_T that ranged between -40 mV and $+200$ mV. The sequencing of the 600 ms pulses and the responses of V_0 measured with the microelectrode are shown in Fig. 3. In general, the skins were polarized first to -20 mV and then to -40 mV followed by polarization of the skins to $+20$ mV and thereafter in increments of 20 mV until the breakdown voltage near 200–220 mV was approached. This procedure was identical to that used previously to generate the steady-state I-V relationships from which the values of E_1 (E_{Na}) were estimated. In the present studies, such I-V plots were made and as the data were similar to those reported previously, only the values of E_1 will be reported (see Table II). It was of interest to observe for the examples shown in Fig. 3 that the values of V_0 approached 0 mV when V_T was approximately 140 mV.

In order to calculate $\%R_0$, the data points relating V_T and V_0 were graphed as shown in Fig. 4. As observed for all negative values of V_0, the relationship between V_T and V_0 was linear and the $\%R_0$ was calculated from the best fit regression line. For the control cell the $\%R_0$ was 73.7% and for the amiloride-treated cell the $\%R_0$ was 95.6%. As the value of V_T is the sum of V_0 and the voltage across the inner barrier, V_i, it was possible to calculate V_i, and this is also shown in Fig. 4 (inner solution positive to cell interior). For the control skin, the value of V_i appeared to increase from approximately 100 mV to 137 mV as the V_T was changed from 0 to 137 mV. Correspondingly, the values of V_0 fell from approximately -100 mV to 0 mV. For the amiloride-treated skin where the short-circuit current was near 0 μA, the V_i was observed to remain essentially constant (130–135 mV) despite a considerable change in the values of both V_T (0–135 mV) and V_0 (-131–0 mV). It was of interest to note that the values of V_T at which V_0 was 0 (near 140 mV) were near the mean values of E_1 estimated previously from the I-V relationships. Accordingly, we labeled this voltage E_1' as shown in Fig. 4. In this way the values of E_1' were defined as the values of V_T when the values of V_0 were 0.

The control values of V_0, $\%R_0$, and E_1' are shown in Table II. The mean V_0 for all skins was −97.5 mV. It was of particular interest to note the very small standard errors for the values of V_0 of each skin. In any particular skin the values of V_0 measured from cell to cell were remarkably similar even when cells

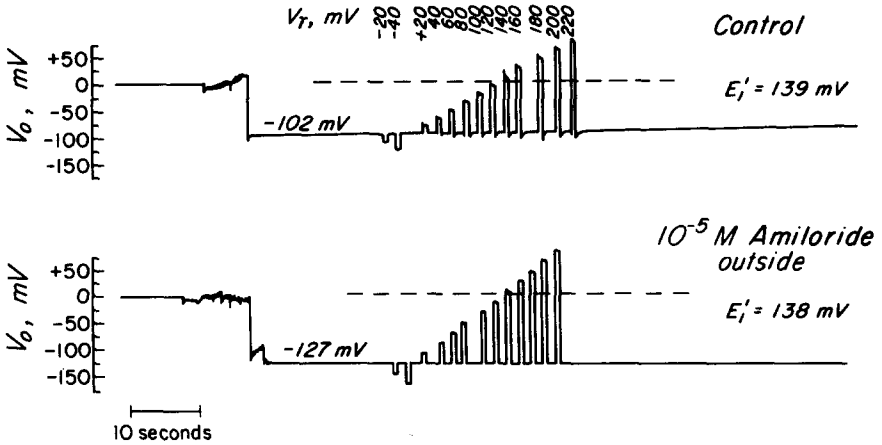


FIGURE 3. Representative cell punctures of control and amiloride-treated skins (redrawn from the original). Appearance of the microelectrode tip at the stratum corneum was signalled by small negative potentials and the appearance of noise. Penetration of a cell was signalled by an abrupt increase in negative potential to a stable value. In some cases, a small tap on the manipulator caused the tip of the microelectrode to seal in place as shown in the lower tracing. When the potential was stable, tapping of the manipulator had no effect on the V_0. Similarly, small advancements of the microelectrode had no effect on the V_0. Although not shown here, continuous advancement of the microelectrode eventually caused the V_0 to fall towards zero with a concurrent decrease of the $\%R_0$ (see text). In every case, the values of V_0 remained negative even when the skins were open circuited for several minutes. In order to determine the relationship between V_T and V_0, the skins were voltage clamped at the values of V_T indicated (−40–220 mV). The values of E_1' were defined as the values of V_T when $V_0 = 0$. Although not shown here, the sequencing of pulses could be repeated in a single cell several times over considerable time intervals (30–90 min) with identical results. In these and previous studies of the I-V relationships, the transepithelial current responses to step changes of the V_T forced by the voltage clamp showed the typical transients. In order to estimate the steady-state values, the pulses were viewed directly with a storage oscilloscope (Tektronix 564B, Tektronix, Inc., Beaverton, Ore.) and the values characteristically appeared stable after a transient of 50–200 ms. In control skins, it was apparent that the V_0 also showed transients that could be appreciable especially when $V_T \gg E_1$. When the skins were treated with amiloride or exposed to low $[Na]_o$, the transients were markedly reduced. The reasons for these differences are not known but would seem to be related at least in part to the magnitude of the transepithelial current flow.

were sampled over the entire area of skin studied. The $\%R_0$ averaged 75.6% and this would indicate that the resistance of the outer barrier was approximately three times larger than the resistance of the inner barrier. Thus the tip of the microelectrode, while recording a markedly negative voltage, had penetrated a

TABLE II

EFFECT OF AMILORIDE ON V_0, $\%R_0$ AND E'_1

	V_0 (mV)			$\%R_0$			E'_1 (mV)		
Skin no.	Control	Amiloride	Change	Control	Amiloride	Change	Control	Amiloride	Change
1	−103.1 ±1.2 (12)	−122.6 ±2.4 (11)	−19.5	75.8 ±1.4 (10,6)	96.2 ±1.7 (16,7)	20.4	134.7 ±1.5 (10,6)	131.0 ±1.5 (16,7)	−3.7
2	−98.4 ±1.1 (9)	−126.1 ±1.0 (14)	−27.7	79.9 ±1.1 (11,7)	98.0 ±.7 (19,11)	22.1	124.6 ±2.1 (11,7)	130.2 ±1.2 (19,11)	+5.6
3	−77.1 ±2.0 (30)	−106.7 ±1.6 (18)	−29.6	61.6 ±3.2 (22,14)	91.4 ±1.7 (5,5)	29.8	117.3 ±3.0 (22,14)	112.6 ±2.7 (5,5)	−4.7
4	−77.4 ±1.1 (9)	−86.9 ±2.4 (7)	−9.5	79.1 ±2.6 (11,6)	91.9 ±0.9 (3,3)	12.8	97.5 ±2.9 (11,6)	90.7 ±4.3 (3,3)	−6.8
5	−104.7 ±2.3 (10)	−119.5 (2)	−14.8	80.3 ±2.5 (21,11)	100.0 (2,2)	19.7	117.1 ±2.4 (21,11)	119.5 (2,2)	+2.4
8	−113.7 ±1.5 (15)	−127.6 ±4.6 (7)	−13.9	66.5 ±1.5 (9,5)	79.1 ±1.7 (9,7)	12.6	159.6 ±2.4 (9,5)	159.6 ±4.7 (9,7)	0.0
9	−107.9 ±2.0 (5)	−118.7 ±1.6 (9)	−10.8	85.7 ±1.5 (10,5)	93.5 ±0.4 (9,5)	7.8	125.9 ±2.7 (10,5)	126.9 ±1.4 (9,5)	+1.0
Mean ± SE (7)	−97.5 ±5.5	−115.4 ±5.4	−18.0 ±3.0	75.6 ±3.2	92.9 ±2.6	17.9 ±2.8	125.2 ±7.2	124.4 ±7.9	−0.9 ±1.7

Values are mean ± SE. The values of $\%R_0$ and E'_1 were determined for each cell. In some cells, these determinations were repeated several times. The values of $\%R_0$ and E'_1 are reported as mean ± SE (number of observations, number of cells).

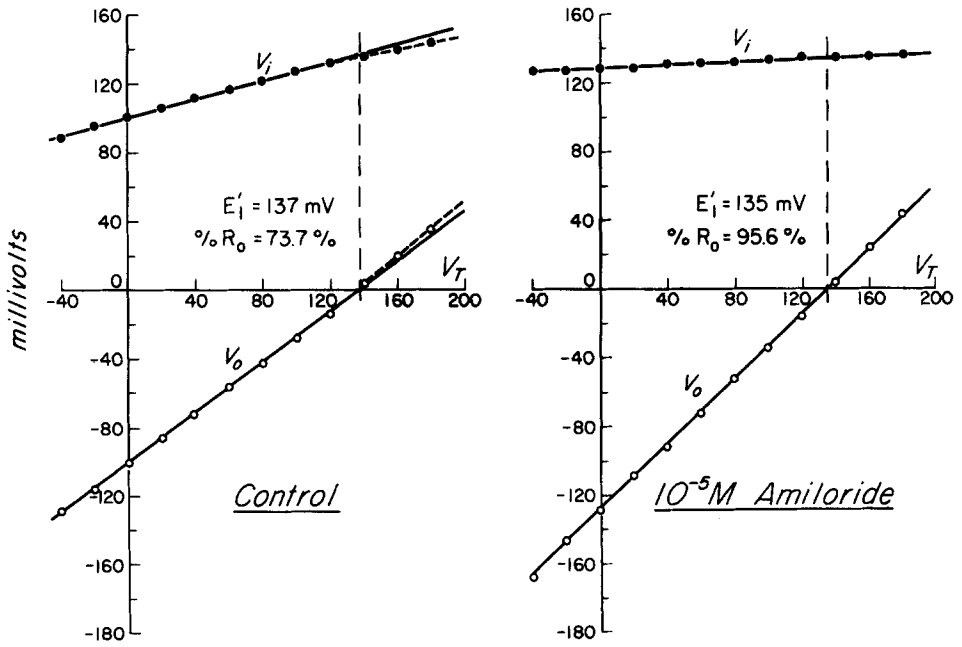


FIGURE 4. Typical relationships between V_T and V_0 for control skins and skins exposed to amiloride. The $\%R_0$ was calculated from the slopes of the lines ($\Delta V_0/\Delta V_T$). The values of E'_1 were calculated at the intercept $V_0 = 0$. V_i was estimated from the difference between V_T and V_0. In control skins a deviation from linearity was observed when the polarity of V_0 was positive as would be expected if electrical rectification is present at the outer membrane (see text). When the skins were treated with amiloride or exposed to 2.4 $[Na]_0$, such deviations were not observed. This is to be expected when the $\%R_0$ approaches 100% as the $\Delta V_T = \Delta V_0$ if $R_0 \gg R_i$.

resistance barrier that accounted for approximately 75% of the total transepithelial resistance. This evidence would support the view that the tips of the microelectrodes had penetrated a barrier beyond the stratum corneum as it has been observed that isolated sheets of stratum corneum possess no potential difference, and moreover possess an insignificant electrical resistance (Helman and Fisher, 1976). Further support for this was available from studies done to assess the effects of amiloride on the V_0, $\%R_0$, and E_1'.

Effects of Amiloride

The procedures were the same as before. Acute experiments were done by adding 10^{-5} M amiloride to the outer solution while a cell was being monitored continuously with a microelectrode. Thereafter, with amiloride still present in the outer solution, several additional cells could be studied and the data com-

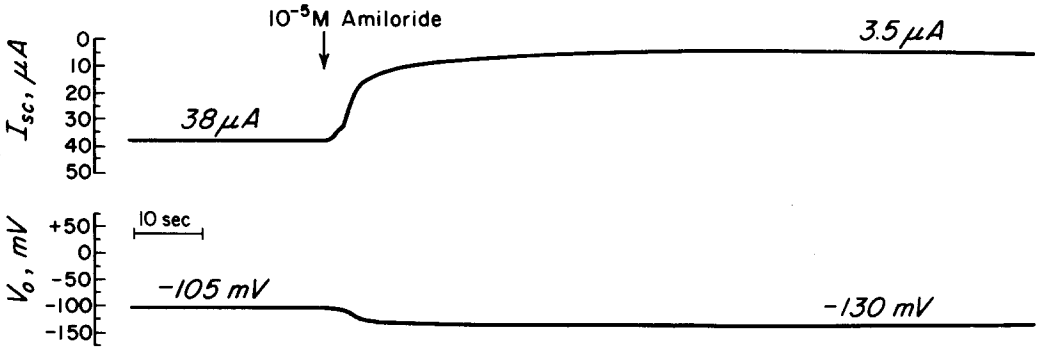


FIGURE 5. Acute effects of 10^{-5} M amiloride on I_{sc} and V_0. The amiloride-containing Ringer was allowed to flow slowly into the outer chamber until no further changes of the I_{sc} or V_0 were observed. The effects were completely reversible. Not shown are the responses of V_0 to voltage clamping of the skin before and after addition of amiloride to the outer chamber. The values of E_1' were 132 mV and 134 mV, respectively, and the $\%R_0$ increased from 79.5% to 97.0%.

pared with the control cells. In some studies, the amiloride was flushed from the outer solution and its effects were observed to be entirely reversible as were the effects of sodium reduction (see below). The results of a typical study are shown in Fig. 5. As the amiloride-containing solution was allowed to flow slowly into the outer chamber so that the microelectrode would not be disturbed, its effect was to decrease I_{sc} from 38 μA to 3.5 μA (0.72 cm^2). Concurrently, the V_0 increased from -105 mV to -130 mV. As noted above, these effects were entirely reversible. V_0 increased from -97.5 to -115.4 for a mean increase of 18 mV. Correspondingly, the $\%R_0$ increased from a mean value of 75.6% to 92.9%. Thus, in the presence of amiloride the outer barrier penetrated by the microelectrode possessed a resistance R_0 approximately 12 times larger than the resistance of the inner barrier, R_i. Interestingly, the values of E_1' averaged near 125 mV and remained unchanged by amiloride despite the considerable changes of the I_{sc} and the resistance of the outer barrier.

Effects of Reducing $[Na]_o$

Studies similar to those above were done by reducing the sodium concentration of the outer solution, $[Na]_o$. As shown in Fig. 6, the I_{sc} fell from 22 μA to 13 μA (0.72 cm^2) as the solution containing 2.4 mEq/liter Na was allowed to flow into the outer solution. Concurrently with the decrease of the I_{sc}, the V_o increased from −108 mV to −122 mV. Upon return to the control solution, the V_o decreased and paralleled the changes of the I_{sc}. A summary of results is shown in Table III. Reduction of $[Na]_o$ from 102.4 mEq/liter to 2.4 mEq/liter caused the

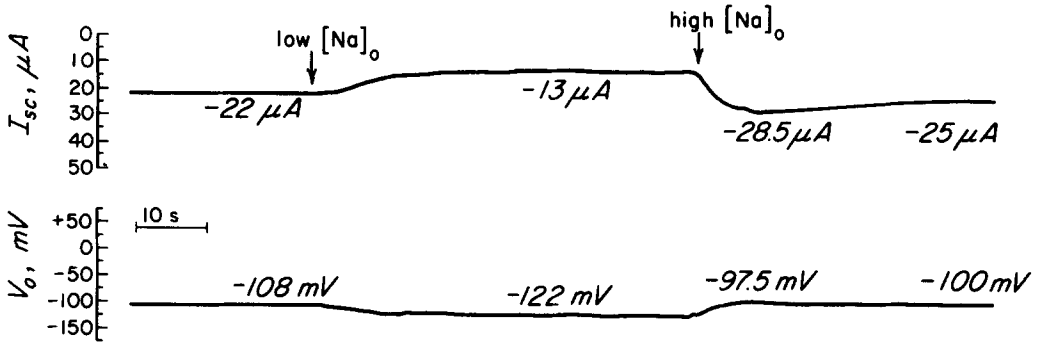


FIGURE 6. Effect of reducing the sodium concentration of the outer solution on I_{sc} and V_o. The I_{sc} fell from −22 μA to −13 μA with a concurrent increase of V_o from −108 to −122 mV. When the $[Na]_o$ was returned to the control value, the I_{sc} and V_o returned towards their control values after a transient period during which the values of V_o and I_{sc} overshot their control values. This was observed consistently.

TABLE III

EFFECT OF $\Delta[Na]_o$ ON V_o, $\%R_o$, AND E_1'

	V_o (mV)			$\%R_o$			E_1' (mV)		
	102.4 $[Na]_o$	2.4 $[Na]_o$	Δ	102.4 $[Na]_o$	2.4 $[Na]_o$	Δ	102.4 $[Na]_o$	2.4 $[Na]_o$	Δ
Mean	−106.9	−125.7	−18.6	79.2	88.8	+12.3	136.5	140.1	+3.6
±SE	±1.4	±1.3	±1.4	±2.6	±4.1	±0.9	±5.9	±5.2	±1.4
(*n*)	(15)	(15)	(15)	(12)	(12)	(12)	(12)	(12)	(12)

V_o to increase from a mean value of −106.9 mV to −125.7 mV. The $\%R_o$ increased concurrently from a mean value of 79.2% to 88.8% and these results are similar to those observed with amiloride. The values of E_1' averaged 136.5 mV and as with amiloride, reduction of $[Na]_o$ had little or no effect on the values of E_1'.

Comparison of E_1 and E_1'

Perhaps the most intriguing and consistent observations made with nonedge-damaged skins are the characteristic "breaks" in the I-V plots (Helman and Miller, 1971). As shown in Fig. 7, two breaks are noted and are labeled E_1 and E_2. It has been suggested that these values of E_1 and E_2 could represent the driving forces for active sodium transport proceeding through independent parallel

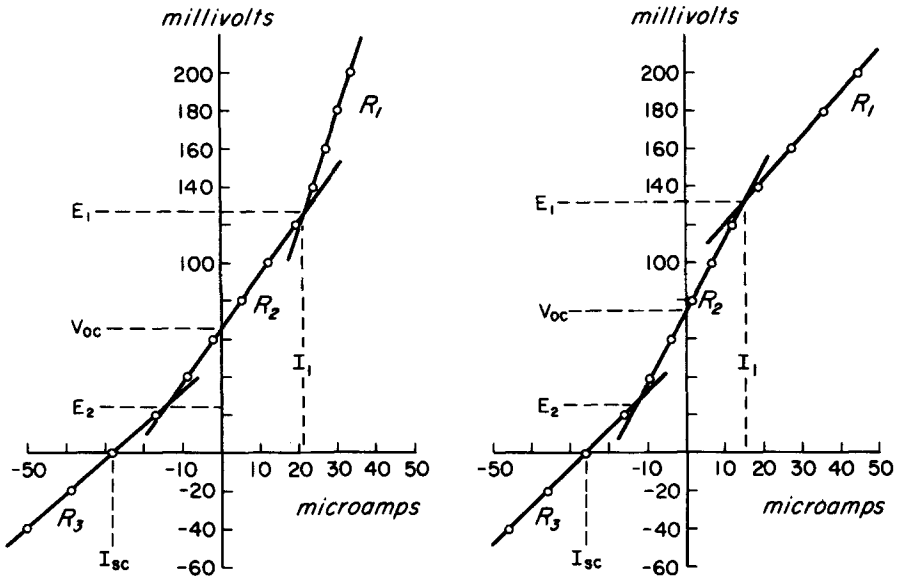


FIGURE 7. Representative I-V plots of frog skin bathed with chloride-Ringer. Characteristically for each skin, the steady-state data points V_T and I_T fall into three regions of linear slope resistance—R_1, R_2, and R_3. During the control period two types of I-V relationships are observed. In most studies (see Table VI) the ratio R_1/R_2 > unity. In some studies the ratio R_1/R_2 < unity. This spontaneous difference between skins is observed especially for skins of southern *R. pipiens berliendieri* bathed with a chloride- or sulfate-Ringer buffered with HCO_3^- and is to be compared with the previous studies of skins of northern *R. pipiens* by Helman and Miller (1971, 1973) where the ratios R_1/R_2 for tissues bathed with a chloride-Ringer buffered with phosphate were consistently greater than unity. The reasons for the difference between skins is unknown, especially for skins bathed with HCO_3^- media. Nevertheless, the values of E_1 have been found to estimate the E_{Na} regardless of the value of ratio R_1/R_2. In this regard, in extensive studies by Macchia and Helman (unpublished observations; Macchia, 1977) it was observed that the slope resistances R_1 and R_2 are increased by amiloride but selectively (with little or no effect on the R_1) so that at concentrations greater than 2.5×10^{-7} M, the I-V relationships appear linear between −40 mV and E_1 (see Fig. 8) and remain so at all concentrations up to 10^{-5} M or higher. Since the slope resistance R_2 is increased in value at a time when slope resistance R_1 remains essentially constant, the ratio R_1/R_2 falls in value regardless of the control value of R_1/R_2. In a sense, the slope R_2 appears to rotate about the coordinate E_1,I_1 with increasing concentrations of amiloride, and as the amiloride has little or no effect on the value of R_1, the ratio R_1/R_2 must decrease. The reason for the insensitivity of the slope resistance R_1 to amiloride is not known. Area = 0.72 cm².

transport pathways, albeit the dominant route of sodium transport for skins bathed with chloride-Ringer (90–95%) is thought to proceed through the "E_1 pathway" (Helman, 1975).[3] In the context of the present studies, it would appear that the microelectrodes penetrated only what are apparently the "E_1 cells."

[3] Helman, S. I., B. M. Koeppen, and D. D. Macchia. Manuscript in preparation.

When studies are done with skins bathed with a chloride-Ringer solution buffered with phosphate, the slope resistance R_1 is greater in value than slope resistance R_2 (Helman and Miller, 1971, 1973). However, when skins are bathed with a chloride-Ringer solution buffered with HCO_3 as done in the present studies, we have observed during the control periods of some skins that the $R_1 < R_2$ (see Fig. 7 and Table VI). Despite this, it has been observed in every study that the values of E_1 are the same as the values of E_{Na} and the quotients E_1/I_1 are the same as the values of R_s (Helman and Miller, 1973; Helman et al., 1975; O'Neil and Helman, 1976; Macchia and Helman, 1974; Macchia, 1977). Thus, it was of considerable interest to observe that the values of E_1' were quantitatively similar to the values of E_1 of the I-V plots. Indeed, when the values of E_1 and E_1' determined simultaneously in 93 cells of nine skins were compared, the mean value of E_1 was 133.1 ± 1.5 mV and that of E_1' was 130.5 ± 1.7 mV. As the ratio of E_1/E_1' was 1.03 ± 0.01 and their difference $(E_1 - E_1')$ was +2.5 ± 1.1, and as these values were not different from 1.00 or 0.0, respectively, it was concluded that the values of E_1 and E_1' were the same and that both provided estimates of the E_{Na} of the sodium pump of E_1 cells. It should be noted that no selection of data was made and we never observed values of V_o that could be suggestive of nor interpreted to mean that the microelectrode had penetrated E_2 cells. If E_2 cells exist, as postulated by this laboratory, they may comprise either a very small population of cells within the outer layers of stratified epithelium or, alternatively, the E_2 cells may be localized elsewhere, perhaps in the cells of the glandular epithelium. In this context, we have studied the I-V relationships of the toad colon and toad urinary bladder (*Bufo marinus*) and both tissues have been observed to possess a break only at voltages E_1 (Macchia and Helman, unpublished observations).

Representative I-V plots are shown in Fig. 8 for skins incubated with low $[Na]_o$. In general, they appeared similar to the control plots possessing breaks at the voltages E_1 and E_2 although the values of I_{sc} are decreased and the slope resistances are increased from the control values. An I-V plot for a skin exposed to 10^{-5} M amiloride in the outer solution is shown in Fig. 9. Although the value of E_1 remained unchanged from the control value, no rectification can be observed at the usual values of E_2. In extensive studies by Macchia and Helman (unpublished observations; Macchia, 1977) it has been observed that amiloride at concentrations $<2.5 \times 10^{-7}$ M causes a selective increase of the values of R_3 so that the I-V plots are linear between −40 mV and E_1 at all concentrations $>2.5 \times 10^{-7}$ M. This has been interpreted to mean that the "E_2 pathway" possesses receptors for amiloride of high affinity that are blocked preferentially at the lower concentrations of amiloride. This idea is supported by (*a*) data on the kinetics of sodium tracer build up (Helman, 1975; see footnote 3), and (*b*) a Scatchard analysis of the binding affinities for amiloride (Macchia and Helman, 1976*b*).

Effects of DNP

As we believe that the values of E_1 represent the driving force for active sodium transport, it was of interest to demonstrate its dependency on metabolism and, perhaps more importantly, to demonstrate that its value could be altered. We

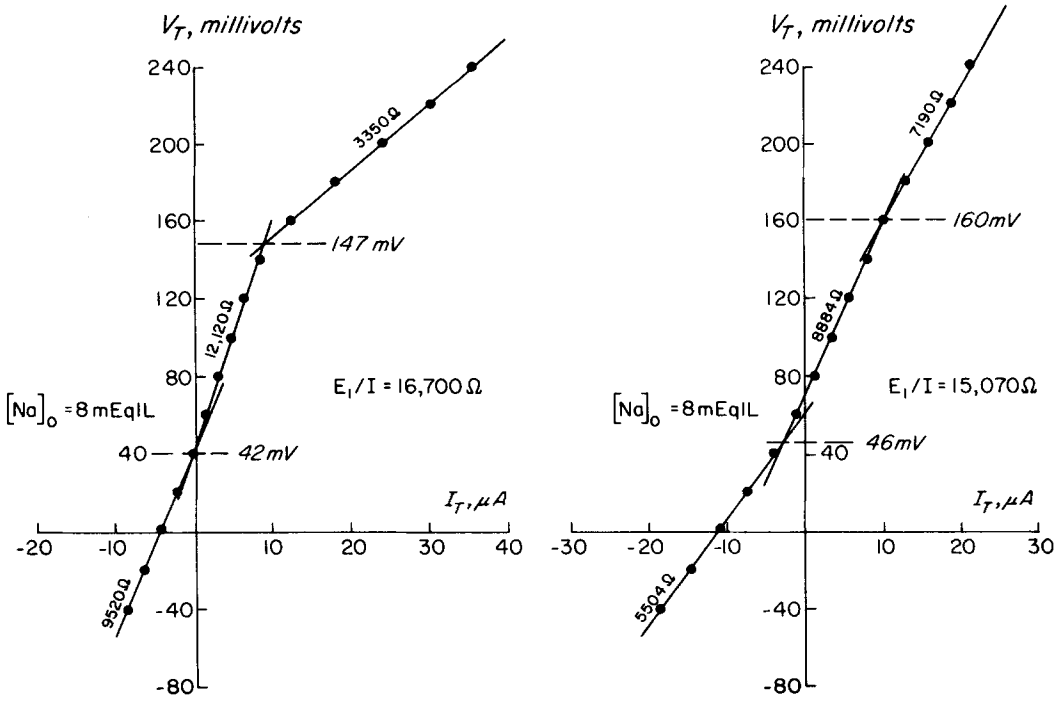


FIGURE 8. I_T-V_T plots of skins bathed with Ringer containing 8 mEq/liter $[Na]_o$. The plots are qualitatively similar to the control plots, but the I_{sc} was decreased from control values and the slope resistances were increased above control values. Values of E_1 and E_2 defined as before. Area = 0.72 cm².

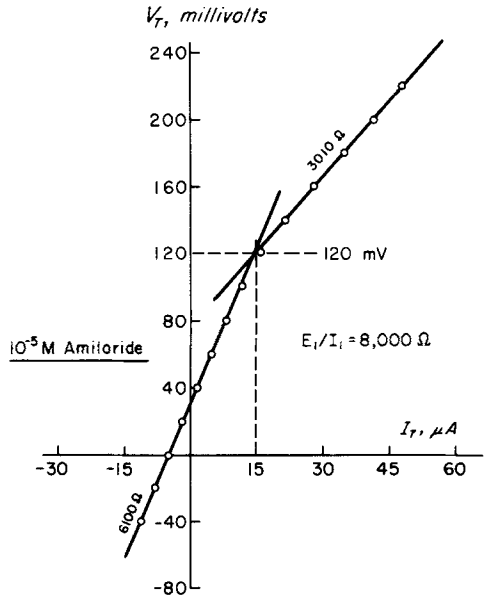


FIGURE 9. I_T-V_T plot of skin exposed to 10^{-5} M amiloride. Note absence of break at E_2 and the linearity of the relationship between −40 mV and +120 mV. E_1 = 120 mV was unchanged by amiloride. $R_1 < R_2$. Area = 0.72 cm².

chose to use 2,4-DNP as we could show in other studies of the I-V relationship that DNP caused a prompt decrease not only of the I_{sc} but also of the values of E_1 (unreported observations). The present studies were done with skins exposed to 10^{-5} M amiloride, as this reduced the I_{sc} to low levels and so permitted the maximum values of V_i and E_1' to be estimated and the effects of DNP on these values to be assessed under conditions where the pump was required to carry out little or no significant transepithelial transport of sodium.

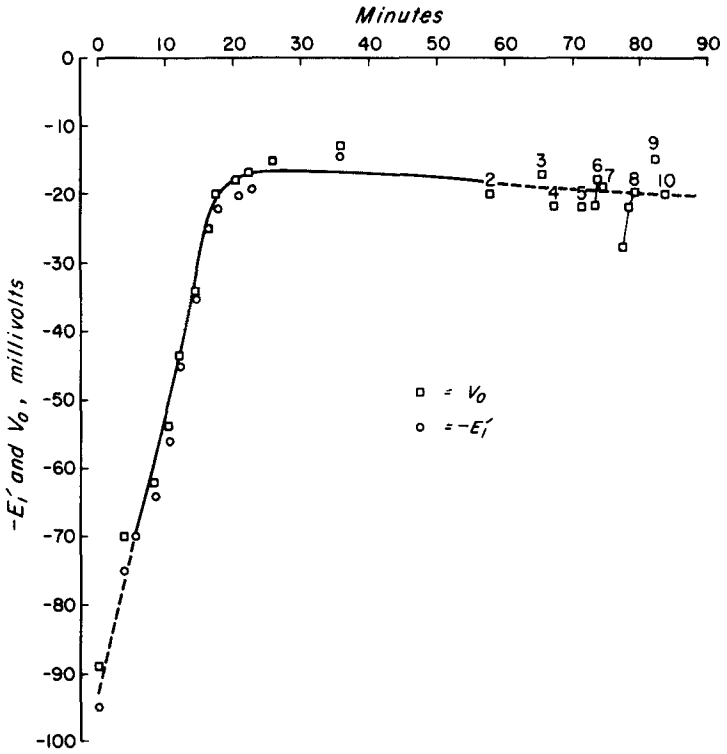


FIGURE 10. Effect of 2,4-DNP (10^{-4} M) on the V_0 and E_1' of an amiloride-treated skin. Within 15-20 min both V_0 and $-E_1'$ fell to near -15 mV (see text and Table V). During the falling phase of the response, the values of $-E_1'$ were slightly larger than the values of V_0. 10 cells were used to obtain data. The first cell was monitored for approximately 50 min followed by punctures of cells nos. 2-10.

The studies were carried out in two ways. First, immediately after addition of 10^{-4} M 2,4-DNP to the inner solution, a single cell was punctured and the values of V_0, $\%R_0$, and E_1' were determined at several time intervals as shown for a typical study in Fig. 10. In this study the values of V_0 fell precipitously, reaching a plateau value near -15 mV within approximately 15 min after addition of DNP to the inner solution. This one cell was monitored for approximately 60 min after which nine additional cells, all possessing similar values of V_0, were punctured. As the skins were continuously short circuited, the values of V_0 were at all times equal in magnitude to V_1. In a second group of studies DNP was

added as before to the inner solution and immediately thereafter several cells were punctured during the falling phase of the V_0. As before, and as can be observed from the results of two representative studies (see Fig. 11), the time course was similar regardless of whether one cell or many cells was used to obtain

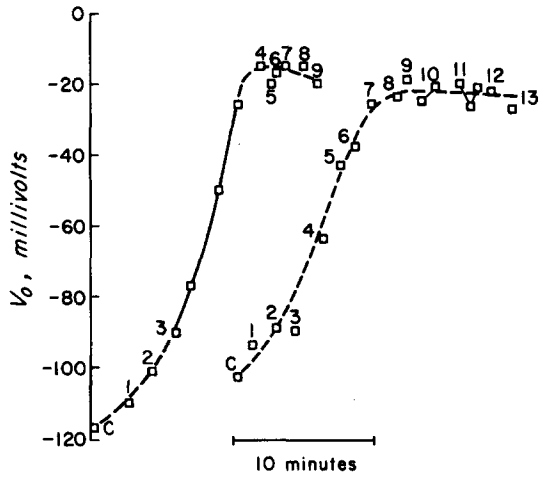


FIGURE 11. Effect of DNP (10^{-4} M) on V_0. Two representative studies are shown. The time course was determined with 9 and 13 cell punctures, respectively. The time course was similar (compare with Fig. 10) regardless of the number of cells punctured during the decreasing phase of the V_0.

TABLE IV

EFFECT OF 2,4-DNP ON V_0, $\%R_0$, AND E_1' OF AMILORIDE-TREATED SKINS

	V_0 (mV)		$\%R_0$		E_1' (mV)	
Skin no.	Amiloride control	DNP	Amiloride control	DNP	Amiloride control	DNP
1	−125.9 ±1.3 (16,7)	−21.1 ±1.1 (15,4)	96.2 ±1.7 (16,7)	80.0 ±2.9 (15.4)	131.0 ±1.5 (16.7)	26.2 ±1.2 (15,4)
2	−127.5 ±1.3 (19,11)	−39.9 ±1.6 (22,7)	98.0 ±0.7 (19,11)	65.9 ±2.6 (22,7)	130.2 ±1.2 (19,11)	61.2 ±1.9 (22,7)
4	−86.9 ±2.4 (7,6)	−31.2 ±2.3 (14,14)	91.9 ±0.9 (3,2)	77.5 ±3.7 (14,14)	90.7 ±4.3 (3,2)	40.5 ±2.4 (14,14)
6	−88.7 ±2.0 (7,7)	−19.3 ±0.8 (18,10)	92.3 ±0.8 (3,3)	67.0 ±5.0 (17,10)	95.3 ±2.0 (3,3)	34.6 ±3.8 (17,10)
7	−98.7 ±3.1 (6,5)	−21.6 ±0.7 (10,7)	92.0 ±2.0 (6,5)	78.0 ±2.7 (7,3)	107.5 ±4.2 (6,5)	25.1 ±1.2 (7,3)
8	−125.9 ±3.4 (9,8)	−43.6 ±1.6 (17,9)	79.1 ±1.7 (9,8)	74.9 ±1.5 (17,9)	159.6 ±4.7 (8,9)	58.2 ±1.9 (17,9)
9	−118.7 ±1.6 (9,7)	−17.8 ±1.4 (5,5)	93.5 ±0.4 (9,7)	73.4 ±4.5 (4,4)	126.9 ±1.4 (9,7)	23.0 ±1.1 (4,4)
Mean ±SE (7)	−110.3 ±6.9	−27.8 ±4.0	91.7 ±2.4	73.8 ±2.1	120.2 ±9.1	38.4 ±5.9

the data. Within 10–15 min after exposure to DNP, the values of V_0 and E_1' fell to low stable values. A summary of results is shown in Table IV. The mean V_0 of the amiloride control group was −110.3 mV and DNP caused its value to decrease to a mean value of −27.8 mV. Values near zero were never observed

even after 2 h of exposure to the DNP. Similarly, the values of E_1', which averaged 120.1 mV before DNP, fell to a mean value of 38.4 mV. The $\%R_o$ fell to 73.8%. This decrease of the $\%R_o$ with DNP could be a result of an increase of the values of R_i and/or a decrease of the values of R_o, and at the present time we are unable to assess this owing to the uncertainty of measuring values of I_{sc} near zero. Additional studies of this and other inhibitors of sodium transport are in progress. At the present time the above data lend direct support to the idea that the values of E_1' and presumably E_{Na} are sensitive to the inhibitory effects of DNP. In a more practical sense the above data also lend support to the idea that the positions of the tips of the microelectrodes were within a compartment beyond the entry step for sodium but before the step (inner barrier) that is responsible for active sodium extrusion.

Specific Resistances of Inner and Outer Barriers

As noted in Materials and Methods, two approaches were used to obtain estimates of R_i and R_o. The first method assumed that $R_{Na} = R_o + R_i$ and that R_{Na} could be calculated from the quotient E_1/I_{sc}. The second method assumed that R_{Na} could be estimated from the difference between R_s and R_{skin} where R_s was estimated from the quotient of E_1/I_1.

The results of a typical study using method A to determine R_{Na}, R_o, and R_i are shown in Fig. 12. During the control period the I_{sc} fell from 29.3 to 16 $\mu A/cm^2$. Concurrently, the R_{Na} increased from 3,980 to 8,269 Ω cm^2 with little or no change in the value of E_1'. From the values of R_{Na} and the $\%R_o$, the specific resistances were calculated and as can be observed in Fig. 12 the increase in R_{Na} could be attributed to an increase in R_o as the values of R_i tended to decrease by approximately 20% from 1,000 to near 800 Ω cm^2. When the values of R_{Na}, R_o, and R_i were determined with method B, the data were similar.

In order to obtain estimates of R_i for all skins and to permit comparison of methods for estimation of R_i, the data for each control period were averaged. The results are shown in Table V. Both methods gave similar values for R_i, although there was considerable variability among skins. The mean R_i was 924 and 1,026 Ω cm^2 for methods A and B, respectively, and these values were not significantly different from each other.

I-V Relationships of Outer and Inner Barriers

In view of the fact that the I-V relationships show electrical rectification, it was of interest to determine the I-V relationships of the inner and the outer membranes separately in order to establish the site of the rectification process.[4] To do

[4] It should be noted that the I_T-V_T relationships of control skins possess regions of slope resistance (R_1, R_2, and R_3) that are strictly linear and when skins are exposed to low concentrations of amiloride, the slope resistance R_2 is linear between −40 mV and E_1. As the values of E_1/I_1 are essentially the same at all concentrations of amiloride up to 10^{-5} M, and whereas the slope resistance R_2 appears linear between −40 mV and the E_1, it would be reasonable to believe that the R_s is also ohmic and linear and independent of the V_T, at least as estimated with 600-ms pulses. It is possible that the breaks at the voltages E_1 and E_2 may be attributed to changes in the values of R_s. However, we are unaware of evidence that supports this view, and we are unable to find data in our studies

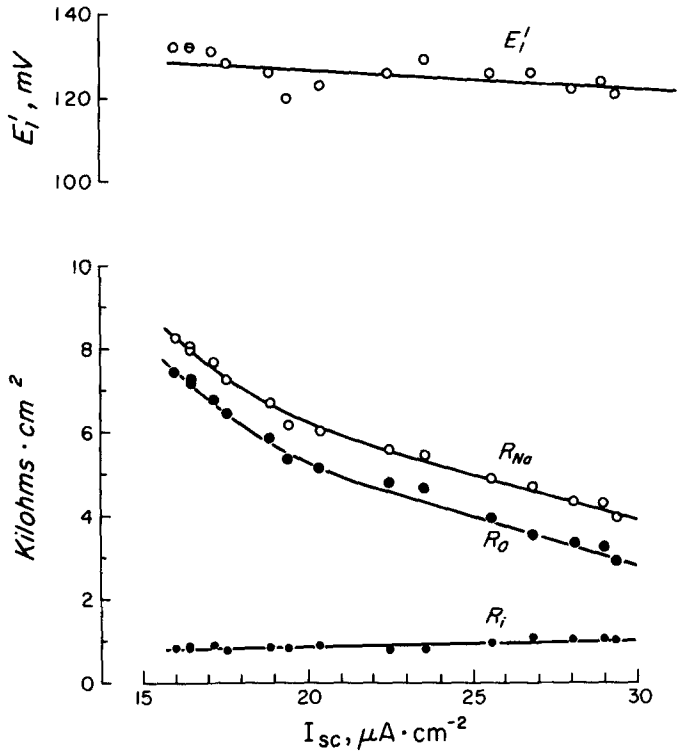


FIGURE 12. Spontaneous changes of R_{Na}, R_o, R_i, and E_1' during the control period of a typical study. Values were calculated with method A (see Materials and Methods). While the value of I_{sc} fell spontaneously during the first 60–120 min of study, the values of R_{Na} increased, and this could be attributed to an increase in the values of R_o. The values of R_i either remained constant or decreased by up to 20%, as shown here. The average values of R_i are reported in Table V.

this, it was necessary to estimate first the current in the shunt pathway, I_s, and then from the difference between I_T (the transepithelial current flow) and I_s to estimate the current flow in the E_1 pathway $I_T^{E_1}$. Accordingly, as R_s could be estimated from the quotient E_1/I_1:

compatible with this view. It may well be that application of prolonged current pulses to the skin or other tissues results in alterations of the electrical parameters as might be expected, especially when the current strengths are large, resulting in large changes of the V_T. For this and other reasons we chose to use pulses 600 ms in duration that appear to have little or no perturbing effect on the electrical characteristics of the skin provided that the V_T range studied is between −40 and +200 mV. Indeed, it would be highly coincidental and fortuitous that the changes in slope resistance should occur by virtue of a change of the R_s so that the values of E_1 and E_1/I_1 would coincide with the values of E_{Na} or R_s estimated with other methods (Macchia and Helman, 1974; Helman et al., 1975; O'Neil and Helman, 1976). It would moreover be fortuitous to find that the change in slope resistance from R_2 to R_1 would occur coincident with a reversal of polarity of the outer membrane if such a change were attributable to a change of the R_s alone. In the context of our past and present studies, the values of E_1 are thought to give the values of E_{Na}.

$$I_s = V_T/(E_1/I_1), \quad (6)$$

and

$$I_T^{E_1} = I_T - I_s. \quad (7)$$

The I-V relationships of the outer membrane were viewed from plots of the corresponding values of V_o and $I_T^{E_1}$. Similarly, plots of the I-V relationships of the inner membrane were generated with the values of $I_T^{E_1}$ and the deviations of voltage from E_1', $(V_i - E_1')$. The data points included in these plots covered the ranges of V_T in regions R_1 and R_2 where the slope resistances were linear. Typical plots for control cells, cells exposed to low $[Na]_o$, and cells treated with amiloride are shown in Figs. 13–15. For each of the 93 cells studied, the R_i was observed to be linear and no evidence was available to indicate that the R_i could account for the electrical rectification of the E_1 pathway. Values of R_i could not be estimated in this way for many cells treated with 10^{-5} M amiloride as the $\Delta I_T^{E_1}$

TABLE V

ESTIMATES OF R_i, $\Omega \cdot cm^2$, OF CONTROL SKINS

Skin no.	Method A	Method B	Δ(B-A)
1	629±35 (10)	670±47 (10)	+41
2	296±19 (11)	396±19 (11)	+100
3	530±27 (17)	798±42 (14)	+268
4	1,501±86 (6)	1,758±22 (7)	+257
5	643±39 (21)	865±63 (20)	+222
7	928±34 (16)	881±35 (12)	−47
8	2,039±127 (9)	2,039±132 (9)	0
9	830±42 (5)	804±42 (4)	−26
Mean±SE (8)	924±203	1,026±200	+102±46

Estimates of R_i determined with methods A and B. Values are mean±SE (number of cells).

was too low to provide resolution of the ΔV_i with ΔV_T. For every cell, it was observed that the rectifying process was located at the outer barrier and this is not surprising in view of the fact that the $\%R_o$ is usually >75%. For negative values of V_o, we defined the resistance of the outer barrier from the linear slope of $\Delta V_o/\Delta I_T^{E_1} = R_o^f$. Correspondingly, for positive values of V_o, the slope resistance $= R_o^b$. For the control cells shown in Fig. 13, the ratios of R_o^b/R_o^f were 1.57 and 0.70, and these observations were consistent with the ratios of R_1/R_2 observed in the I_T-V_T plots. A summary of control parameters is shown in Table VI. The values of R_i were similar to those reported in Table V. The R_o^f/R_i ratio averaged 4.82 and thus the R_o^f accounted for approximately 83% of the transcellular resistance. The ratios R_o^b/R_o^f were generally greater than unity and corresponded to the ratios of R_1/R_2 observed in the I-V plots of the entire skin. When the values of R_1/R_2 were less than unity, the ratios of R_o^b/R_o^f were also less than unity.

The effects of low $[Na]_o$ on the R_o^f, R_o^b, and R_i are illustrated in Fig. 14 and summarized in Table VII for cells where control and low $[Na]_o$ data were available for the same cells. For the example shown in Fig. 14, the value of R_i

remained the same when $[Na]_o$ was decreased. The control R_o^b/R_o^f ratio was 1.11 (5,340/4,800) and this ratio fell to 0.51 due predominantly to an increase in the value of R_o^f from 4,800 Ω to 14,300 Ω. Indeed, reduction of $[Na]_o$ and amiloride caused selective increases in the values of R_o^f with lesser, if any, effects on the values of R_o^b. Accordingly, it is reasonable to believe that the rectification observed in the I-V plots of the intact skins is attributable to the ratio of R_o^b/R_o^f at

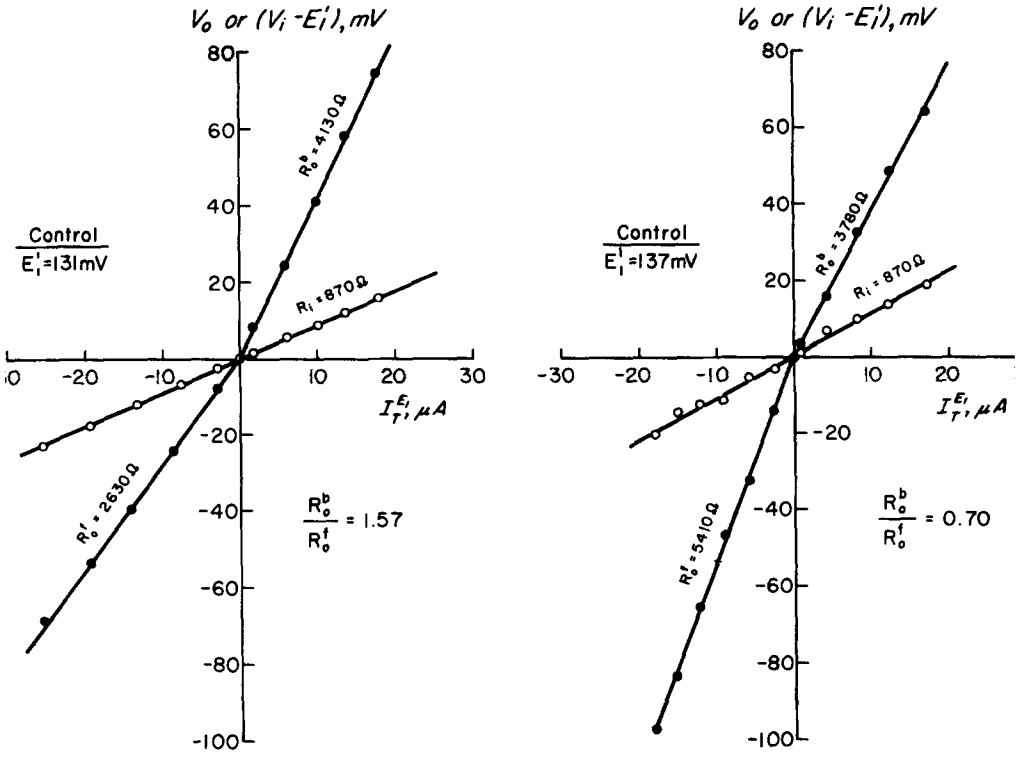


FIGURE 13. I-V plots of inner and outer membranes of two control cells. Note that the R_i is linear (see also Figs. 14 and 15). Note also that $R_o^b > R_o^f$ or $R_o^f > R_o^b$. For the examples shown here $R_o^b/R_o^f = 1.57$ and 0.70. For the range of values where the V_o is either positive or negative, the slopes appeared linear and the values of R_o were calculated with linear regression analysis. Area = 0.72 cm^2.

the outer barrier of the cells. These findings taken with those above are of special interest in view of the fact that the changes of slope resistance coincided with reversal of polarity at the outer barrier of the cells. If this reversal of polarity can be taken to indicate a reversal of the current flow at the outer barrier, then several possibilities need to be resolved before it is possible to understand the meaning of the ratio R_o^b/R_o^f. It would be reasonable to believe that when V_o is negative, Na ions carry the current into the cells from the outer solution. However, when V_o is positive, it is possible that Na ions also carry the current from the cell to the outer solution but owing either to its lower

intracellular concentration or to differences in the interactions of the Na ions with the transport mechanisms (number or binding characteristics) of the outer barrier, the values of $R_o^b \neq R_o^f$. It is also possible owing to the high potassium concentration of the intracellular compartment that K ions conduct the current through the outer barrier when the V_o is positive. Alternatively, reversal of the V_o may alter the transport characteristics of the outer membrane directly via electrical field effects. Resolution of these possibilities amongst others will require systematic study.

Finally, the effects of amiloride on the R_o^b, R_o^f, and R_i are illustrated in Fig. 15

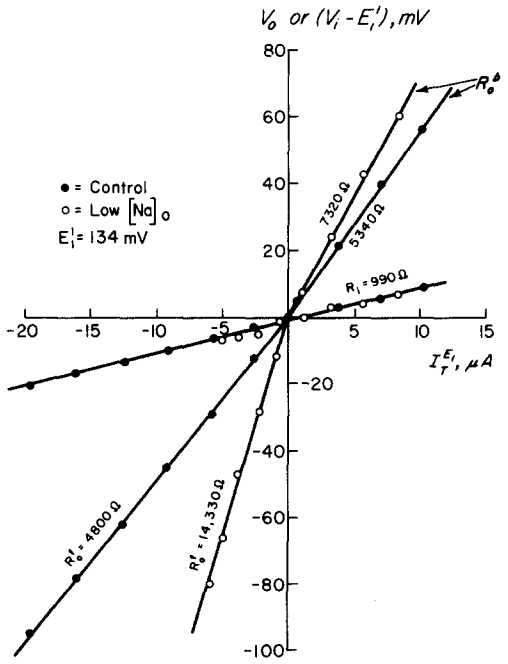


FIGURE 14. Plots are similar to that of Fig. 13 and show the effect of low $[Na]_o$ on the R_o^f, R_o^b, and R_i of a single cell. The R_o^f increased approximately threefold with a relatively small effect on R_o^b. No effect on R_i was observed. Ratio R_o^b/R_o^f fell from 1.11 to 0.51. Area = 0.72 cm².

and summarized in Table VIII. The data were gathered from studies done with skins exposed to 10^{-5} M amiloride or to lesser concentrations near 10^{-6} M. In general, the data were similar to those observed for reduction of $[Na]_o$. The dominant effect of amiloride was to increase the R_f^o to values that in some cells exceeded 100,000 Ω cm² with little or no effect on the R_i or on R_o^b. For those cells where $I_{sc} \simeq 0$ and where $\Delta I_T^{E_t'}$ was essentially zero, values of R_i could not be calculated and this is reflected in the data of Table VIII for the values of R_o^f/R_i and R_i.

DISCUSSION

In order to understand the mechanisms by which epithelial tissues such as the frog skin are capable of actively reabsorbing sodium, the electrochemical poten-

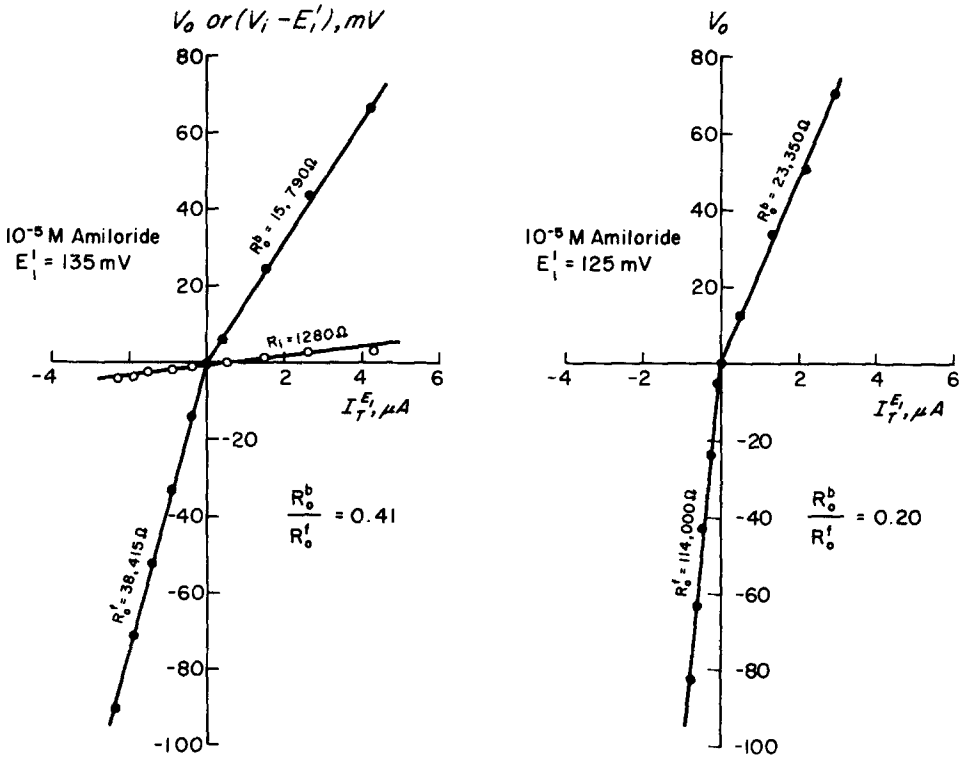


FIGURE 15. Typical plots for amiloride-treated cells. R_0^b/R_0^f = 0.41 and 0.20. When the values of R_0^f exceed 50,000–100,000 Ω, the small $\Delta I_T^{E_i'}$ do not permit the slope R_i to be estimated, since V_i remains essentially constant.

TABLE VI

CONTROL PARAMETERS OF R_0^b, R_0^f, AND R_i

Skin no.	R_1/R_2	R_0^b/R_0^f	R_0^f/R_i	R_i
	$I_T - V_T$			Ω·cm²
1	1.15±0.016 (9)	1.41±0.06 (5)	2.87±0.29 (5)	997±116 (5)
2	1.26±0.016 (11)	1.53±0.05 (7)	4.49±0.93 (7)	531±57 (7)
3	1.26±0.043 (12)	1.57±0.12 (9)	2.41±0.42 (9)	713±49 (9)
4	1.11±0.012 (3)	1.23±0.05 (2)	2.77±0.44 (2)	2,675±356 (2)
5	1.13±0.006 (15)	1.38±0.06 (12)	4.63±0.65 (12)	1,191±143 (12)
6	1.00 (7)	0.84	11.1	1,733
7	0.94±0.071 (11)	0.91±0.04 (8)	6.95±0.54 (8)	1,125±56 (8)
8	0.93±0.087 (17)	0.74±0.03 (14)	3.35±0.37 (14)	1,883±150 (14)
Mean±SE (8)		1.20±0.12	4.82±1.04	1,356±248

Summary data for control cells. Values of R_1/R_2 were estimated from the I_T-V_T plots. Values of R_i were estimated from the slope $\Delta V_i/\Delta I_T^{E_i'}$, and this constituted a third, but not independent method for estimating R_i. The ratios were calculated from the slope resistances (see Fig. 13). Values are mean ± SE (number of cells).

tials encountered during its transport must be known. The best-known model, proposed by Koefoed-Johnsen and Ussing in 1958, viewed this process as occurring at two membranes in series, each possessing asymmetric characteristics. The outer barrier was thought to behave as a passive Na electrode and the inner barrier was thought to behave as a K electrode but also to possess a neutral Na-K exchange pump. According to this model, it was thought that the transepithelial voltage could be ascribed to two series voltage steps at the outer and inner barriers. While under open-circuit conditions, the cell interior would possess a positive polarity relative to the outer solution, having a magnitude somewhere

TABLE VII

EFFECT OF Na REDUCTION ON R_o^f, R_o^b, AND R_i

Control				low $[Na]_o$				low $[Na]_o$/Control			
I_{sc}	R_o^b/R_o^f	R_o^f/R_i	R_i	I_{sc}	R_o^b/R_o^f	R_o^f/R_i	R_i	I_{sc}	R_o^b/R_o^f	R_o^f/R_i	R_i
$\mu A \cdot cm^{-2}$			$\Omega \cdot cm^2$	$\mu A \cdot cm^{-2}$			$\Omega \cdot cm^2$				
16.0	0.71	9.9	723	5.1	0.41	23.2	717	0.32	0.58	2.3	0.99
15.4	0.79	6.5	1,025	5.7	0.48	20.7	825	0.37	0.61	3.2	0.80
16.1	0.89	7.6	905	3.5	0.49	22.3	771	0.22	0.55	2.9	0.85
27.4	0.72	2.1	1,898	18.8	0.54	3.0	1,845	0.69	0.75	1.4	0.98
32.2	1.11	4.8	714	9.9	0.51	11.7	881	0.31	0.46	2.4	1.23
21.4	0.84	6.2	1,051	8.6	0.49	16.2	1,008	0.38	0.59	2.5	0.97
±3.5	±0.07	±1.3	±217	±2.8	±0.02	±3.9	±211	±0.08	±0.05	±0.3	±0.07

Effects of low $[Na]_o$ on R_i, R_o^b, and R_o^f. Data are summarized for five cells where values were determined in control Ringer and after reduction of $[Na]_o$. Little or no change in R_i was observed. As R_o^f was selectively increased in value (see Fig. 14), the ratio R_o^f/R_i increased. The ratio R_o^b/R_o^f fell.

TABLE VIII

EFFECT OF AMILORIDE ON R_o^b, R_o^f, AND R_i

	I_{sc}	R_o^b/R_o^f	R_o^f/R_i	R_i
	$\mu A \cdot cm^{-2}$			$\Omega \cdot cm^2$
Control (Tables I and VI)	37.1±8.6	1.20±.12	4.8±1.0	1,356±248
Partial amiloride (~10^{-6} M)	11.1±0.4 (12)	0.48±.02 (16)	7.5±1.2 (16)	1,354±184 (12)
10^{-5} M amiloride	2.2±0.4 (13)	0.31±.02 (13)	25.7±2.7 (6)	973±186 (7)

Effects of amiloride on R_i, R_o^b, and R_o^f. Amiloride had little or no effect on R_i. Accordingly, with increased inhibition of the I_{sc}, the R_o^f/R_i increased and the R_o^b/R_o^f decreased. These effects are similar to those observed with low $[Na]_o$.

between 0 and the V_{oc}. Under short-circuit conditions, while sodium was actively transported across the tissue, the cell interior would become negative owing to an ohmic voltage developed at the outer barrier in excess of the concentration potential for Na at the outer barrier. Support for these ideas was obtained in studies of skins where microelectrodes were used to penetrate the tissue from its outer surface. (Engbæk and Hoshiko, 1957; Ussing and Windhager, 1964; Whittembury, 1964; Cereijido and Curran, 1965; Biber and Curran, 1970; Rawlins et al., 1970). In general, the positive voltage steps were identified and localized in the cells of the stratum germinativum and these data seemed to confirm the model as well as the observations of MacRobbie and Ussing (1961) that localized the transporting cells at the level of the stratum germinativum.

Several significant observations have led some investigators to question this particular model for the frog skin. For example, it has been known from the time of Krogh that frogs take up Na from pondwater containing as little as 10^{-5} M Na (Krogh, 1937; Krogh, 1938). Biber and Curran (1970) and others (Biber et al., 1966; Morel and Bastide, 1965; Zerahn, 1969) calculated that the electrochemical gradients for Na entry were inadequate for a process of passive Na entry and it was proposed that Na uptake by the skin at the outer border could be active. Moreover, Cereijido and Curran (1965) in their microelectrode studies of the skin could not resolve their observations that the outer and inner barriers did not behave as Na and K electrodes, respectively, and they were compelled to question the validity of the model of Koefoed-Johnsen and Ussing (1958). Indeed, in accord with the findings of Frazier and Leaf (1963) and Bricker et al. (1963), they suggested that the PD across the inner barrier may arise by an electrogenic mechanism of active sodium extrusion. In more recent studies of the toad bladder, Finn (1974, 1976) has also questioned the validity of the Koefoed-Johnsen-Ussing model, as he was able to show that changes of the V_T with several experimental manipulations could not be explained with this model. It is clear from the results of the present studies of frog skin, which are in accord with the findings of Nagel (1975) and those of frog skin done by Reuss and Finn (personal communication), that the data presented here are not in accord with the earlier literature nor do they support the model of Koefoed-Johnsen and Ussing. Consequently, it is important to review the pieces of evidence that may be important in evaluating the present work.

First, we know of no case where previous investigators have not observed a negative potential upon initial penetration of the skin from the outer surface. This negative potential ranging from a few millivolts to near −50 mV has been ascribed to the cells of the stratum corneum (Engbæk and Hoshiko, 1957; Nunes and Lacaz Vieira, 1975), although such potentials were not associated with the penetration of a high-resistance outer barrier. In the present studies we also observed small negative potentials upon initial contact of the microelectrode tip with the stratum corneum, but this was always followed by a marked increase in negative potential that remained stable for long periods of time. Moreover, this abrupt change in potential was associated with the tip having passed through a high-resistance outer barrier of some 75% of the transcellular resistance, and indeed, amiloride and low $[Na]_0$ increased the resistance between the tip and the outer solution to >90% of the total resistance. These data are difficult to attribute to simple artifact as, in any one skin, the consistency and repeatability of the punctures were, if anything, surprising to us even when several electrodes were purposely used to obtain data.

The data of the present studies and those of Nagel could be considered to be controversial if it remained impossible to provide a reasonable explanation for the lack of observation of markedly negative stable potentials by other investigators. If it were recalled that the values of V_0 approached 0 mV as the values of V_T approached E_1, it would be difficult at best to detect the negative potentials when the values of open-circuit voltage V_{oc} were especially high and perhaps similar in value to E_1. In this regard, it was noted consistently while we reviewed the literature that the skins studied by others were punctured while open circuited

and, moreover, the skins studied possessed mean open-circuited voltages unusually high in value, averaging 129 mV in the studies of Ussing and Windhager (1964), 117.5 mV in the studies of Cereijido and Curran (1965), 107 mV in the studies of Whittembury (1964), ranging between 73 mV and 145 mV in the studies of Engbæk and Hoshiko (1957), and between 85 mV and 95 mV in the studies of Rawlins et al. (1970). Accordingly, under these conditions, the values of V_o would approach 0 mV, and consequently, as the microelectrode penetrated the outer barrier, the voltage observed would be small and perhaps only a few millivolts even though the resistance between microelectrode tip and outer solution could have exceeded 75% of the transcellular resistance. With deeper penetrations of the skin, it would not be unreasonable to expect that leaks could develop around the microelectrode as its shank was advanced through the high-resistance outer barrier. Indeed, in order for the tip to reach the cells of the stratum germinativum, the microelectrode must be advanced at least 50 μm or more through the outer barrier, and this undoubtedly would result in a significant leak around the microelectrode. If this occurred, an artifactual shunt pathway would be created along the track of the microelectrode and this pathway, behaving as a current sink adjacent to active tissue as described by Lindemann (1975), would result in the microelectrode tip recording a more positive potential whose magnitude would in part depend on the voltage divider ratio between the tip and the respective bathing solutions. We believe that the above explanation may account at least in part for the difference in observations between present and past studies. Although there may be reasons other than the above, we are not able to conceive of a simpler explanation.

It is also of interest to note that evidence has accumulated to support the view that the cells of the stratum granulosum are responsive to those procedures that are known to affect the transepithelial transport of Na (Voûte and Ussing, 1968; Dörge et al., 1974; Helman and Fisher, 1977). It would thus be reasonable to think that the abrupt changes in potential and the associated penetration through a high-resistance barrier could be attributed to the cells of the stratum granulosum. Direct confirmation of this with dye injections or similar procedures has not yet been done. Any attempt to advance the microelectrode into deeper layers of the skin resulted consistently in a marked reduction in the magnitude of the negative potential, and this was associated with a decrease of the $\%R_o$ as though the microelectrode had broken through the high-resistance barrier, creating a shunt around the microelectrode. In view of this, we made no attempt to study the skins with deep microelectrode penetrations. The fact remains that with the microelectrodes recording the large negative potential values, the outer barrier responded predictably and consistently to amiloride and reduction of $[Na]_o$. There are, of course, other procedures to be investigated that are known to affect the outer barrier, and such studies are in progress.

A second criterion for assessing tip localization would be to assess the inner barrier and those procedures that might influence its physiology. Clearly, under short-circuit conditions the magnitudes of V_o and V_i must be identical, and for control skins the V_i averaged near 100 mV. Unquestionably, Na extrusion must be active, whereas sodium entry into the pool monitored by the microelectrode is

most likely passive (see below). When the transepithelial voltage, V_T, was increased towards the spontaneous values of open-circuit voltage and beyond, the magnitude of V_i increased and the magnitude of V_o decreased. It should be noted that even at the open-circuit voltage the polarity of V_o was always negative. The polarity of the outer barrier was changed only when the values of V_T exceeded the values of E_1'.[5] We found this observation of considerable interest by virtue of the fact that the point of reverse biasing of the outer barrier (cell positive to outer solution) coincided with the values of V_T (E_1') that were observed previously to give the value of the E_{Na} of the sodium pump (Helman et al., 1975). This coincidence of observations seems extremely fortuitous, especially in view of the fact that the values of E_1 are known to vary considerably between skins and in parallel with the values of E_1' observed in the present studies. In other studies it has been observed that the values of E_1 remain essentially constant when skins are exposed to amiloride or to reduction of $[Na]_o$ and this, too, was observed for the values of E_1'. On the premise that these results could have been entirely fortuitous, we tested further to see whether 2,4-DNP could affect the values of E_1'. Indeed, this inhibitor caused a prompt decrease in its value. Accordingly, these data as well as those above should permit the conclusion that the tips of the microelectrodes were most likely in a compartment separating inner and outer barriers of the transport mechanism, and moreover, that the driving force for sodium transport (E_1) is located at this inner barrier.

Perhaps the most interesting inhibitor of sodium transport to be tested is ouabain owing to its specificity for the Na-K-ATPase. In other studies where the effects of ouabain on the values of E_1 were determined, we could demonstrate only a small decrease (~20%) of its value (unpublished observations). This at first may appear surprising, as it did to us. In recent microelectrode studies of skins by Helman and Nagel (unpublished observations), the values of V_o and E_1' have been observed to fall in magnitude by approximately 30–35% within 10 min after addition of ouabain at 10^{-4} M to the inner solution. In this regard the effects of ouabain on the E_{Na} appear similar, as assessed from measurements of the E_1 or from measurements of the E_1'.

Electrical Model of the Active Pathway (E_1 Pathway)

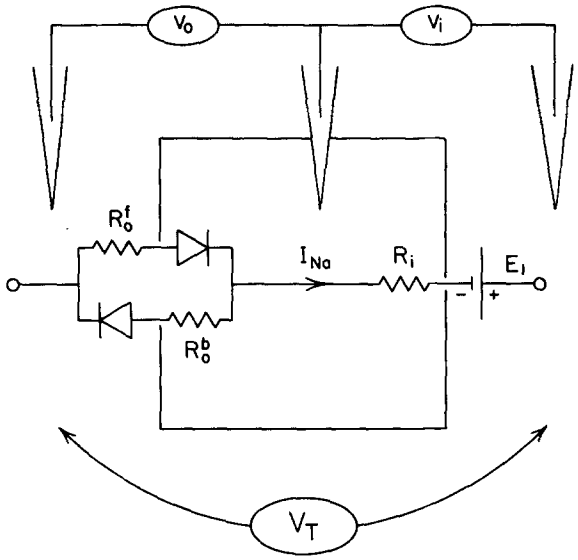
In the context of the present and our past studies, we considered a simple electrical model of the "E_1 pathway" that encompassed and summarized several observations. The model is shown in Fig. 16. The inner barrier is modelled with a Thévenin equivalent consisting of an emf of value E_1' and an internal resistance R_i. The voltage at the inner barrier is:

$$V_i = E_1' - I_T^{E_1} R_i. \tag{8}$$

When $I_T^{E_1}$ is reduced to zero such as by amiloride or reduction of external $[Na]_o$,

[5] Values of V_T greater than E_1 would never be observed under physiological conditions, as the maximum values of $V_{oc} = E_1$ when $R_s \to \infty$. Thus, in the physiological sense, the values of R_o^b have no physiological significance, and the values of V_o would be negative when $V_T < E_1$. It is convenient, however, that the existence of the electrical rectifying property of the R_o permits estimation of the E_{Na} and R_s in studies of the I-V relationships.

the V_i will approach the value of E_1' as observed in the present studies. Under conditions of short-circuiting ($V_T = 0$) the V_o must be equal in magnitude to V_i, and thus the values of V_o become more negative approaching the magnitude of E_1' when $I_T^{E_1}$ is decreased towards zero. When the cell is voltage clamped at the



$$V_T < E_1 \qquad I_{Na} = (E_1 - V_T) / (R_o^f + R_i)$$
$$V_T = E_1 \qquad I_{Na} = 0$$
$$V_T > E_1 \qquad I_? = (V_T - E_1) / (R_o^b + R_i)$$

FIGURE 16. Electrical equivalent circuit of the E_1 pathway of active sodium transport. The inner barrier is modelled with a Thévenin equivalent consisting of emf, E_1, and resistance, R_i. The outer barrier is modelled with two parallel conductance pathways, each consisting of a resistance and an ideal diode. When V_o is negative ($V_T < E_1$) sodium ions carry the current from the outer solution via R_o^f to the intracellular compartment. When $V_T > E_1$ and the V_o is positive, current is carried via R_o^b from cell to outer solution. Since $R_o^b \neq R_o^f$, the outer barrier rectifies the current flow. Although the following is speculative, K may contribute to the R_o^b owing to its high intracellular concentrations (see text for other possibilities). Not shown is the parallel shunt pathway $R_s = E_1/I_1$.

values of $V_T = E_1'$, $I_T^{E_1}$ will be reduced to zero as is the voltage V_o at the outer barrier, and moreover, the $V_i = E_1'$.

As noted above, it has been observed consistently that the I-V relationships possess linear regions of slope resistance. As the change in slope resistance coincides with $V_o = 0$ and as the outer barrier possesses 75% or more of the transepithelial resistance, it would be reasonable to believe that the rectification observed in the I-V plane is attributable to the ion transport properties of the outer barrier. We have modelled the outer barrier with ohmic resistances and

ideal diodes, as shown in Fig. 16. When V_0 is negative Na enters the cell from the outer solution via the equivalent resistance, R_0^f. However, when the V_0 is positive ($V_T > E_1'$), current flows from cell to outer solution via the equivalent resistance, R_0^b. If the ratio of resistances R_0^f/R_0^b is different from unity, then the slope resistances R_1 and R_2 observed in the I_T-V_T plots would also be different from unity. If we first consider graphically the simplest case, $R_S = \infty$, an I_T-V_T plot of the E_1 pathway would appear as shown in Fig. 17 A where we assumed that $R_0^b >$

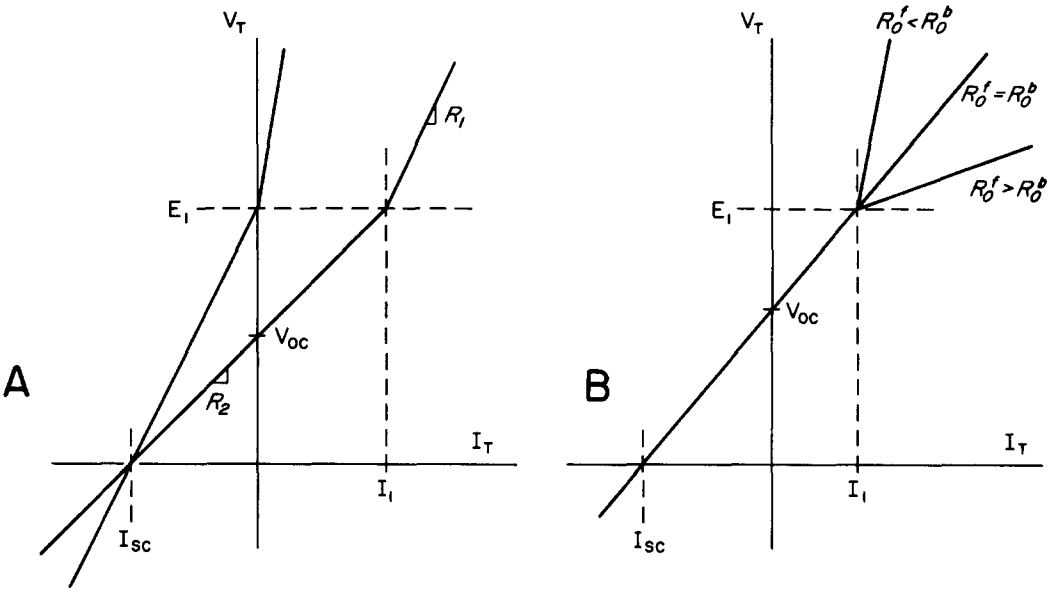


FIGURE 17. Theoretical plots of I_T-V_T relationships for tissues that can be modelled with a single active transport pathway and a parallel ohmic shunt pathway. Illustrated in A are the I-V relationships for $R_s = \infty$ and for $R_s = E_1/I_1$ and where it is assumed that E_1 is constant. Note that with a decrease in value of R_s, the slope resistances R_1 and R_2 also decrease in value with no change of the I_{sc}. Illustrated in B are the I-V relationships expected when the ratios of R_0^f/R_0^b are less than, equal to, or greater than unity. When $R_s \ll R_0$, as may be the case for leaky epithelia or edge-damaged frog skin, the I-V plots tend toward linearity and despite the existence of rectification in the active path, it would be difficult at best to observe the rectification in the I-V plots as is the case especially for edge-damaged skin (Helman and Miller, 1973).

R_0^f. When $V_T = E_1'$, the transepithelial current flow of the active pathway is zero. At voltages of $V_T < E_1'$ slope resistance $R_2 = R_0^f + R_i = R_{Na}$. When $V_T > E_1'$, the V_0 of the outer membrane becomes positive, and consequently current flow through the outer membrane is conducted via R_0^b. If $R_0^b > R_0^f$, then $R_1 > R_2$ since $R_1 = R_0^b + R_i$. If we consider the I-V relationship of such a model in the presence of a shunt pathway, the general appearance of the plot would be similar with rotation of the slope resistances about I_{sc}. The values of E_1 would remain constant and the $R_1 > R_2$. When the $V_T = E_1$, current flow in the E_1 pathway would be zero as before and the current I_1 flowing through the tissue would be

conducted through the shunt pathway alone. Accordingly, the quotient E_1/I_1 would estimate the values of R_s. In general, as shown in Fig. 17 B where the values of R_o^b may be either less than, equal to, or greater than the values of R_o^f, slope resistance R_1 would correspondingly be less than, equal to, or greater than the slope resistance, R_2.

Perhaps the unexpected finding of rather well-defined "breaks" in the I-V plots can be attributed to the fact that the values of E_1' as observed in the present studies from cell to cell are so similar. Indeed, if the values of E_{Na} of each cell showed considerable scatter, the I-V plots would be expected to be curvilinear over similar ranges of voltage. As this is not the case, the coincidence of well-defined "breaks" at E_1 (and perhaps at E_2) are not at all surprising in view of the homogeneity of the values of E_1'. It should be noted, however, that the values of E_1 defined at the intersection points of slope resistances R_1 and R_2 are subject to some uncertainty, as there is undoubtedly a curvilinearity in the transition zones between R_2 and R_1. Although we have not attempted to define the exact nature of this, it would seem clear that within a maximum range of 20 mV corresponding to the V_T sampling interval, the values of E_1 are likely to be within ±5–10 mV of the estimated value, thus representing an error of approximately ±4% to ±8%. If we take the estimates of V_o and E_1' and their standard errors as indicative of the homogeneity of the properties of the transporting cells, then uncertainties in the range of 5–10 mV in the estimates of E_1 would not be unreasonable.

According to the model proposed by Koefoed-Johnsen and Ussing, the potential difference at the outer barrier would be expected to be cell interior positive owing to a diffusion potential for Na ($P_{Na} >> P_K$ and P_{Cl}). However, Schultz (1972) has noted that for an epithelium possessing a parallel shunt pathway, the V_o could be negative depending on the relative values of R_o, R_i, and R_s and the emfs E_o and E_i of inner and outer barriers. If we examine the electrical equivalent circuit shown in Fig. 18 where inner and outer barriers are modelled with Thévenin equivalents E_o, R_o and E_i, R_i, respectively, it can be shown that the V_o is:

$$V_o = E_o\left[1 - \frac{R_o}{R_o + R_i + R_s}\right] - E_i\left[\frac{R_o}{R_o + R_i + R_s}\right]. \quad (9)$$

When $R_o >> R_i + R_s$, the $V_o \cong -E_i$. Accordingly, when the $\%R_o$ is high, as is the case for frog skin, the voltage V_o of short-circuited skins ($R_s = 0$) would approach the value of E_i, and so the values of V_o would be essentially uninfluenced by the values of E_o, especially when skins are treated with amiloride or exposed to low $[Na]_o$. Thus, within the framework of this model system, the magnitude and polarity of V_o would depend to a large extent on the ohmic properties of the outer barrier and to a lesser extent on the values of E_o.

Nevertheless, it would be expected that the values of V_o would in part reflect a chemical potential for Na, especially if $P_{Na} \gg P_{Cl}$ and P_K and provided that the outer barrier is symmetrical with regard to its permeation properties for ions. If the $P_{Na} \ll P_{Cl}$ and P_K for amiloride-treated skins, it remains possible that the V_o (and E_o) could reflect a significant contribution of the K and Cl concentration gradients to the V_o at the outer barrier. This seems unlikely, however, as the

response of the V_0 to amiloride or reduced $[Na]_0$ is the same when the skins are bathed with sulfate-Ringer (Nagel, personal communication). Moreover, elevation of K at constant low $[Na]_0$ and substitution of isethionate for Cl are without significant effects on the V_0 (unpublished observations). Thus, whereas it is reasonably certain that the V_0 depends to a large extent, if not alone, on the ohmic properties or the electrical resistance of the outer barrier, the dependency of the V_0 on the chemical potentials of Na, K, and Cl is rather uncertain. Indeed, the findings of the present studies, taken at face value, indicate that within conceivable experimental errors of 5–10 mV, the values of E_0 can be con-

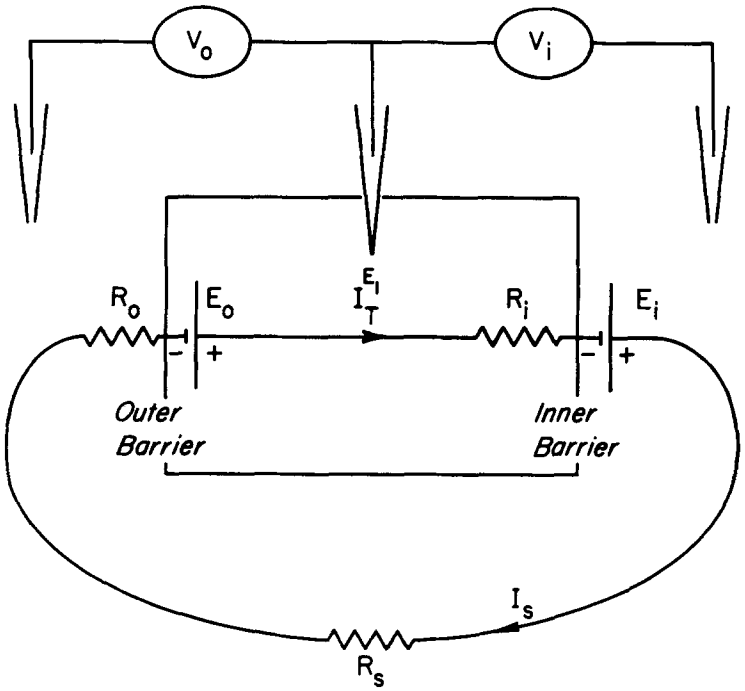


FIGURE 18. Electrical equivalent circuit of a transporting cell modelled with Thévenin equivalents E_0, R_0 and E_1, R_1 at the outer and inner barriers, in parallel with a shunt resistance R_s.

sidered to be essentially zero. Accordingly, if chemical potentials exist, especially for Na, the outer barrier appears to behave as though the "effective" chemical potential is near 0 mV. In this regard, it was observed that the values of E_1 and E_1' were the same, and in view of our previous findings that E_1 estimates the E_{Na} (Helman and Miller, 1974; Helman et al., 1975; O'Neil and Helman, 1976), it would be reasonable to believe that the transepithelial driving force for active sodium transport is developed at the inner barrier alone. Consequently, the finding that the $V_0 = 0$ mV when $V_T = E_1'$ would be consistent with the notion that the outer barrier behaves in a purely passive way with an effective chemical potential not different from zero. If so, this latter finding is difficult to resolve in classical terms.

At least two possibilities might be considered to explain the observation that $V_0 = 0$ at $V_T = E_{Na}$. First, it may be that the Na transport pool is so small that (its concentration is always similar to that of the outer solution and) changes of concentration occur rapidly during voltage-clamping of the skins. Although it would seem that such a possibility is rather unlikely, it cannot be completely excluded at this time. Second, it remains possible that an asymmetry of the outer barrier perhaps associated with differences of the binding affinities for Na and K, or other unknown factors, could exist that might in addition relate to the nature of the electrical rectifying properties of this barrier. Clearly, an adequate description of this barrier will require a much more detailed analysis than is currently available to understand the nature of the entry process and of the intracellular Na transport pool. As an empirical observation, it is presently of interest that the values of E_1' estimate the E_{Na} when the $V_0 = 0$, at least under the conditions of the present studies. This requires that the E_0 or chemical potential of the outer barrier contribute little or nothing to the transcellular flow of current. If the $I_T^{E_1'}$ is indeed zero when $V_0 = 0$, a zero current flow could arise from either a zero value of Na influx or equally well from a 1:1 exchange of Na for K yielding a net charge transfer of zero. If such speculation is tenable, it may well be that even at $V_0 = 0$ mV, Na entry continues but is balanced by an equal loss of K. Clearly, with the V_0 near -100 mV or greater, the electrical gradient would favor Na entry to the extent that K loss could be considered to be negligible. However at $V_0 = 0$ mV, in the absence of an electrical gradient, it would be of interest to evaluate the exchange of Na and K at the outer barrier.

Despite the above uncertainties, the origin of the V_0 cannot be ascribed alone to a simple diffusion potential for Na. Indeed, it is known that Na entry is a saturable process (Kirschner, 1955) mediated by a sodium-selective carrier-like transport process (Cuthbert and Shum, 1974).

In summary, the results of the present studies are in accord with the results of previous studies of the I-V relationships of the frog skin. Not resolved are the mechanism whereby the sodium ion gains entry into the cell or the factors that contribute to the electrical conductance of the outer membrane, especially with regard to the process of electrical rectification. To resolve these issues, it will be necessary to study the inner and outer barriers separately, as it is clear that the membranes are coupled to each other at least electrically, and that changes in either of these barriers alone or together will affect the transepithelial transport of sodium.

We thank Dr. Wolfram Nagel for providing us with a copy of his manuscript on measurements of intracellular potentials of the frog skin.

This study was supported by United States Public Health Service Research Grant AM 16663 from the National Institute of Arthritis, Metabolism, and Digestive Diseases.

Received for publication 29 March 1976.

REFERENCES

BIBER, T. U. L., R. A. CHEZ, and P. F. CURRAN. 1966. Na transport across frog skin at low external concentration. *J. Gen. Physiol.* **49**:1161–1176.

BIBER, T. U. L., and P. F. CURRAN. 1970. Direct measurement of uptake of sodium at the outer surface of the frog skin. *J. Gen. Physiol.* **56**:83–99.

BRICKER, N. S., T. BIBER, and H. H. USSING. 1963. Exposure of the isolated frog skin to high potassium concentrations at the internal surface. I. Bioelectric phenomena and sodium transport. *J. Clin. Invest.* **42**:88–99.

CEREIJIDO, M., and P. F. CURRAN. 1965. Intracellular electrical potentials in frog skin. *J. Gen. Physiol.* **48**:543–557.

CEREIJIDO, M., and C. A. ROTUNNO. 1968. Fluxes and distribution of sodium in frog skin. A new model. *J. Gen. Physiol.* **51**(5, Pt. 2):280s–289s.

CHOWDHURY, T. K., and F. M. SNELL. 1965. A microelectrode study of electrical potentials in frog skin and toad bladder. *Biochim. Biophys. Acta.* **94**:461–471.

CHOWDHURY, T. K., and F. M. SNELL. 1966. Further observations on the intracellular electrical potential in frog skin and toad bladder. *Biochim. Biophys. Acta.* **112**:581–583.

CUTHBERT, A. W., and W. K. SHUM. 1974. Amiloride and the sodium channel. *Naunyn-Schmiedebergs Arch. Pharmakol.* **281**:261–269.

DÖRGE, A., K. GEHRING, W. NAGEL, and K. THURAU. 1974. Localization of sodium in frog skin by electron microprobe analysis. *Naunyn-Schmiedebergs Arch. Pharmakol.* **281**:271–280.

ENGBAEK, L., and T. HOSHIKO. 1957. Electrical potential gradients through frog skin. *Acta Physiol. Scand.* **39**:349–355.

FINN, A. L. 1974. Transepithelial potential difference in toad urinary bladder is not due to ionic diffusion. *Nature (Lond.).* **250**:495–496.

FINN, A. L. 1976. Changing concepts of transepithelial sodium transport. *Physiol. Rev.* **56**:453–464.

FRAZIER, H. S., and A. LEAF. 1963. The electrical characteristics of active sodium transport in the toad bladder. *J. Gen. Physiol.* **46**:491–503.

HELMAN, S. I. 1975. Evidence for two sodium transport pools in isolated frog skin. Abstracts of the 5th International Biophysics Congress, Copenhagen.

HELMAN, S. I., and R. S. FISHER. 1977. Stratum corneum of frog skin: Inferences for studies of Na entry and transport pool. *Am. J. Physiol.* **232**:C37–C44.

HELMAN, S. I., and D. A. MILLER. 1971. *In vitro* techniques for avoiding edge damage in frog skin. *Science (Wash. D. C.).* **173**:146–148.

HELMAN, S. I., and D. A. MILLER. 1973. Edge damage effect on electrical measurements of frog skin. *Am. J. Physiol.* **225**:972–977.

HELMAN, S. I., and D. A. MILLER. 1974. Edge damage effect on measurements of urea and sodium flux in frog skin. *Am. J. Physiol.* **226**:1198–1203.

HELMAN, S. I., R. G. O'NEIL, and R. S. FISHER. 1975. Determination of the E_{Na} of frog skin from studies of its current-voltage relationship. *Am. J. Physiol.* **229**:947–951.

KIRSCHNER, L. B. 1955. On the mechanism of active sodium transport across the frog skin. *J. Cell. Comp. Physiol.* **45**:61–87.

KOEFOED-JOHNSEN, V., and H. H. USSING. 1958. The nature of the frog skin potential. *Acta Physiol. Scand.* **42**:298–308.

KROGH, A. 1937. Osmotic regulation in the frog (*R. esculenta*) by active absorption of chloride ions. *Scand. Arch. Physiol.* **76**:60–74.

KROGH, A. 1938. The active absorption of ions in some freshwater animals. *Z. Vgl. Physiol.* **25**:335–350.

LINDEMANN, B. 1975. Impalement artifacts in microelectrode recordings of epithelial membrane potentials. *Biophys. J.* **14**:1161–1164.

MACCHIA, D. D. 1977. Characterization of the I-V relationships of the toad urinary bladder, toad colon, frog skin and turtle urinary bladder. Ph.D. Dissertation. University of Illinois, Urbana, Ill.

MACCHIA, D. D., and S. I. HELMAN. 1974. Determination of the shunt resistance (R_s) and the emf of the sodium pump (E_{Na}) in frog skin. *Fed. Proc.* **33:**1251.

MACCHIA, D. D., and S. I. HELMAN. 1976*a*. Anion permeability of nonedge-damaged frog skin. *Biophys. J.* **16**(2, Pt. 2):131 a.

MACCHIA, D. D., and S. I. HELMAN. 1976*b*. Binding characteristics of amiloride in frog skin as determined by Scatchard analysis. *Physiologist.* **19:**281.

MACROBBIE, E. A. C., and H. H. USSING. 1961. Osmotic behavior of the epithelial cells of frog skin. *Acta Physiol. Scand.* **53:**348–365.

MOREL, R., and F. BASTIDE. 1965. Action de l'ocytocine sur la composante active du transport de sodium par la peau de grenouille. *Biochim. Biophys. Acta.* **94:**609–611.

NAGEL, W. 1975. Reinvestigation of intracellular PD of frog skin epithelium. Abstracts of the 5th International Biophysics Congress, Copenhagen. P-147.

NUNES, M. A., and F. LACAZ VIEIRA. 1975. Negative potential level in the outer layer of toad skin. *J. Membr. Biol.* **24:**161–181.

O'NEIL, R. G., and S. I. HELMAN. 1976. Influence of vasopressin and amiloride on the shunt pathway of frog skin. *Am. J. Physiol.* **231:**164–173.

RAWLINS, F., L. MATEU, F. FRAGACHAN, and G. WHITTEMBURY. 1970. Isolated toad skin epithelium. Transport characteristics. *Pfluegers Arch. Eur. J. Physiol.* **316:**64–80.

SCHULTZ, S. G. 1972. Electrical potential differences and electromotive forces in epithelial tissues. *J. Gen. Physiol.* **59:**794–798.

USSING, H. H., and E. E. WINDHAGER. 1964. Nature of shunt path and active sodium transport path through frog skin epithelium. *Acta Physiol. Scand.* **61:**484–504.

USSING, H. H., and K. ZERAHN. 1951. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta. Physiol. Scand.* **23:**110–127.

VOÛTE, C. L. and H. H. USSING. 1968. Some morphological aspects of active sodium transport. The epithelium of the frog skin. *J. Cell. Biol.* **36:**625–638.

WHITTEMBURY, G. 1964. Electrical potential profile of the toad skin epithelium. *J. Gen. Physiol.* **47:**795–808.

ZERAHN, K. 1969. Nature and localization of the sodium pool during active transport in the isolated frog skin. *Acta Physiol. Scand.* **77:**272–281.