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Protocol-Dependence of Equivalent Circuit Parameters of Toad Urinary Bladder

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Summary. Determinations of current-voltage relationships are widely employed in the characterization of epithelial sodium transport. In order to determine the protocol dependence of transport parameters in the toad urinary bladder, studies were carried out in the presence and absence of amiloride, an inhibitor of active sodium transport. With symmetric positive and negative perturbations of the transepithelial electrical potential difference $\Delta\psi$ ($0 \rightarrow \pm 100$ mV) for 30 sec, the amiloride-sensitive current-voltage ($i^a - \Delta\psi$) relationship was near linear over the range $-75 \rightarrow +100$ mV, indicating constancy of the conductance κ^a and the apparent electromotive force "E_{Na}", lumped parameters of the standard electrical equivalent circuit model of the active transport system. With a reverse protocol ($\pm 100 \rightarrow 0$ mV) or 15 min perturbations the $i^a - \Delta\psi$ relationships were highly nonlinear. Nonlinearity reflected voltage dependence of parameters: perturbations that increased active transport decreased "E_{Na}" and increased κ^a, as evaluated from 10 sec perturbations of $\Delta\psi$; slowing of active transport produced the converse changes. These effects are usefully analyzed in both quasi-steady states and true steady states by means of a detailed equivalent circuit incorporating the significant ionic currents across each plasma membrane. Precise understanding of the significance of κ^a and "E_{Na}" will require characterization of the partial ionic conductances on perturbation of $\Delta\psi$.

In attempts to understand the nature of active transport processes it often proves useful to interpret mechanisms in terms of the parameters of a simplified representation of the transport system. Thus in the analysis of active sodium transport in anuran epithelia it is common to evaluate the conductance and electromotive force of sodium transport of an electrical equivalent circuit [8, 16, 19, 21, 22, 24, 33, 38, 40, 42, 50, 51, 57]. More recently, active transport has been interpreted in terms of a linear nonequilibrium thermodynamic (NET) formulation [7, 27–29, 36, 43, 52]; the pertinent parameters are then the phenomenological coefficients and affinity of the driving reaction.

Whatever formulation is employed, the evaluation of tissue parameters requires perturbation of the system and the observation of resultant effects on transport and/or metabolism. Most often this is accomplished by altering the transepithelial electrical potential difference $\Delta\psi$. In many of our previous studies of active sodium transport in the toad bladder, current-voltage relationships were determined some 5–30 sec after perturbing $\Delta\psi$ [24, 42, 44]. In studies of the associated oxidative metabolism the requirement for constant slopes in plots of O_2 tension *vs.* time necessitated perturbations for 6 min [27–29, 36, 43, 52]. In order to assure the achievement of steady states, others have employed periods as long as 60 min [26].

Because of the possible influence of the specific protocol on the parameters under study, we have here carried out a systematic study of the dependence of the electrical current on the transepithelial electrical potential difference, varying the extent, duration, and sequence of perturbations of $\Delta\psi$. It was found that long term perturbations of $\Delta\psi$ induced pronounced effects on both the amiloride-sensitive conductance κ^a and the apparent electromotive force of sodium transport "E_{Na}", lumped parameters of the standard equivalent circuit. The effects were further analyzed by means of a detailed equivalent circuit incorporating flows of Cl and/or K, as well as that of Na, across the serosal plasma membrane. This analysis clarified the relationship between parameters mea-

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sured in quasi-steady states and the parameters of true steady states.

(Some of this material has been presented briefly in a symposium in honor of Professor H.H. Ussing [15].)

Materials and Methods

I. General Methods

Urinary bladders of female toads (*Bufo marinus*, Dominican Republic, National Reagents, Bridgeport, Conn.) were studied by methods described previously [18, 46]. Hemibladders were mounted in modified Ussing-Zerahn chambers of 7.1 cm^2 cross-sectional area. Each reservoir was filled with 20 ml Na-Ringer solution. The electrical potential difference $\Delta\psi$ ($\psi_{serosal}-\psi_{mucosal}$) was regulated with a voltage clamp, correcting automatically for solution resistance, and the current I, mucosa to serosa, was recorded continuously. Tissues were mounted carefully without stretching and selected to minimize edge damage. Thus a hemibladder was rejected if the open-circuit potential was less than 40 mV 30 min after mounting. In 45 of the hemibladders used, initial mean open-circuit potential was 57.3 ± 2.6 (SE) mV and mean short-circuit current I_0 was 25.1 ± 1.4 (SE) $\mu A\cdot cm^{-2}$ chamber area. Mean passive conductance κ^p was 0.245 ± 0.015 (SE) mmho $\cdot cm^{-2}$ chamber area and the mean ratio of amiloride-sensitive to total conductance $\kappa^a/\kappa\equiv(\kappa-\kappa^p)/\kappa$ was 0.435 ± 0.019 (SE) ($n=40$). (Values of κ^p were substantially higher than in Erlij's study [14].)

II. Protocols for Current-Voltage Relationships

Series 1. (*a*) Following equilibration at "short-circuit" ($\Delta\psi=0$) for 30 min or longer, $\Delta\psi$ was perturbed at 30-sec intervals with pulses of 30 sec duration in the sequence $\Delta\psi=0$, +25, 0, −25, 0, +50, 0, −50, 0, +75, 0, −75, 0, +100, 0, −100, 0 mV, with the current I being recorded continuously.

(*b*) Series 1*b* differed from 1*a* in that the sequence of perturbations was $\Delta\psi=0$, +100, 0, −100, 0, ... +25, 0, −25, 0 mV.

Series 2 and 3. With perturbations of $\Delta\psi$ at intervals of 5 min (series 2) or 15 min (series 3) the sequence was 0, +25, −25, ..., +100, −100 mV (*a* series) or +100, −100, ..., +25, −25, 0 mV (*b* series), respectively.

III. Conductance Determinations

(*a*) The total conductance κ was determined from the change in current caused by 10-sec pulses, changing the potential by ±20 mV automatically at desired intervals: $\kappa=-\Delta I/\Delta(\Delta\psi)$. Similar (1–10 sec) perturbations are commonly employed in the evaluation of equivalent circuit parameters [5, 9, 16, 18, 23, 24, 29, 39, 42, 44, 45, 49, 57]. While measurements at 10 sec will eliminate capacitive effects, they may reflect the influence of polarization and conductance changes [16–20, 38, 40]. However, slight deviation from square wave responses, often demonstrable at rapid chart speed [24], were of minor significance here, as compared with the magnitudes of the conductance changes induced by the 5–15 min perturbations under study.

(*b*) "Passive conductance" κ^p was determined at the end of the experiment by the addition of amiloride to the mucosal medium to a concentration of 10^{-4} M. This brought the short-circuit current I_0 close to zero (2.5 ± 0.4 (SE) % ($n=29$) of the value prior to amiloride). By extrapolation of the plot of I_0 *vs.* κ, κ^p was evaluated as the intercept in the ordinate at $I_0=0$ [24]. (This value differs insignificantly from the conductance measured in the presence of fully inhibitory concentrations of amiloride, $-(\delta I/\delta\Delta\psi)^A$.) In control studies κ^p was determined shortly after mounting, and 2 hours later in 7 hemibladders. The ratio of the final to the initial value was 1.02 ± 0.09 (SE) ($n=13$), similar to results found earlier [24, 26]. Previous studies had demonstrated that amiloride does not change the passive conductance, as evaluated from serosal to mucosal sodium tracer fluxes [24].

The effect of long term perturbations of $\Delta\psi$ on κ^p was evaluated in hemibladders in which active sodium transport had been nearly abolished by exposure of the mucosal surface to 10^{-4} M amiloride. Following equilibration at $\Delta\psi=0$ for 10–20 min, $\Delta\psi$ was maintained at −50, −75, −100, or +100 mV for 30 min, the current I being recorded continuously. In several tissues the conductance was determined directly by perturbing $\Delta\psi$ by ±20 mV relative to the baseline level for periods of 10 sec about every 5 min.

The effect of $\Delta\psi$ on κ^p in the presence of active transport was studied according to the following protocol: Following equilibration at open circuit for 30 min, paired hemibladders from the same animal were voltage clamped at $\Delta\psi=0$. Thirty min later about 150 μCi of ^{36}Cl was added to the mucosal solution of tissue *a* and to the serosal solution of tissue *b*. Mucosal and serosal solutions were then sampled at 15-min intervals, permitting the evaluation of influx $\vec{J}_{Cl}$ in tissue *a* and of efflux $\vec{J}_{Cl}$ in tissue *b*. After 45 min $\Delta\psi$ was clamped at +75 mV for tissue *a* and at −75 mV for tissue *b*. Sampling was continued as previously so as to provide 3 measurements at $\Delta\psi=0$ mV, followed by 6 measurements at +75 and −75 mV in tissues *a* and *b*, respectively.

Solutions were assayed for radioactivity on a Packard Tricarb liquid scintillation counter, employing standard techniques. The unidirectional fluxes (mucosal to serosal $\vec{J}$ and serosal to mucosal $\overleftarrow{J}$) were computed from the quotients of the appropriate isotope flux and the corresponding gradient of specific activity.

(*c*) The amiloride-sensitive conductance κ^a was defined as the difference of the total and passive conductance:

$$\kappa^a\equiv\kappa-\kappa^p\equiv-[(\delta I/\delta\Delta\psi)-(\delta I/\delta\Delta\psi)^A]. \quad (1)$$

IV. Measurement of Amiloride-Sensitive Current I^a

I^a was evaluated as the difference of the total current and the "passive" current [20, 24, 26, 28]:

$$I^a\equiv I-I^p=I+\kappa^p\Delta\psi. \quad (2)$$

V. Determination of the Apparent Electromotive Force of Sodium Transport "E_{Na}" and $(\Delta\psi)_{I^a=0}$

"E_{Na}" was evaluated from the relationship

$$I^a=\kappa^a(\text{"}E_{Na}\text{"}-\Delta\psi) \quad (3)$$

at each setting of $\Delta\psi$ (Fig. 7). $(\Delta\psi)_{I^a=0}$ was taken as the intercept of the least squares line for unreduced $I^a-\Delta\psi$ data (Figs. 1, 2, 4).

VI. Estimation of "Unstirred" Layer Thickness

The thickness l of the poorly stirred layer at the mucosal surface was estimated from the half time $t_{1/2}$ for abolition of the short-circuit current following exposure to 10^{-4} M mucosal amiloride, by use of the equation of Diamond [13]:

$$l=\sqrt{Dt_{1/2}/0.38}. \quad (4)$$

D, the diffusion coefficient for amiloride, was taken as 6.35 $\times 10^{-6}$ cm^2 sec^{-1}, based on interpolation in Stein's curve relating D to molecular weight [48].

VII. Materials

The composition of the the sodium-Ringer solution was (in mM/liter): 116 Na, 2.5 K, 0.9 Ca, 118 Cl, and 2.4 HCO_3 (pH 7.6, 233 mOsm/Kg H_2O). The composition of the phosphate-Ringer solution was (in mM/liter): 119 Na, 3.5 K, 0.9 Ca, 118 Cl, 2.4 HPO_4, and 0.57 H_2PO_4 (pH 7.2, 220 mOsm/Kg H_2O).

^{36}Cl was obtained from New England Nuclear Co., Boston, and amiloride was obtained from Merck, Sharp, and Dohme Co.

VIII. Analysis of Data

Parameters were expressed as the arithmetic mean ± standard error of the mean (SEM). Results in paired hemibladders were compared by Student's t test; p was the value of the null hypothesis. Linearity of current-voltage relationships was evaluated by least squares analysis of reduced data, giving $y=a+b(\pm SE)x$, the correlation coefficient r, and the SE of estimate S_{xy}. Similar analysis of unreduced data in a near-linear range of interest provided the mean slope and mean x-intercept $(\Delta\psi)_{I^a=0}$ [47].

Results

I. Potential-Dependence of the Amiloride-Sensitive Current

The dependence of the electrical current I on the transepithelial electrical potential difference $\Delta\psi$ was determined employing six sequences differing in the order and duration of perturbations of $\Delta\psi$. Active transport was then eliminated by the use of amiloride, permitting the determination of the passive conductance κ^p, and the amiloride-sensitive current I^a (*see* Methods, Eqs. (1) and (2)). In order to combine observations in several tissues, weighting each equally, it was convenient to relate the value of I^a at a given setting of $\Delta\psi$ to the initial value at $\Delta\psi=0$ and that at $\Delta\psi=100$ mV to give the reduced quantity $i^a_{\Delta\psi}$ $=(I^a_{\Delta\psi}\ i^a_{\Delta\psi}=(I^a_{\Delta\psi}-I^a_{100})/(I^a_0-I^a_{100})$; thus the reduced quantities i^a_0 (initial) and i^a_{100} at 0 and 100 mV are assigned the values 1.0 and zero, respectively. This facilitated comparison of the effects of various protocols on the linearity of the current-voltage relationships (Table 1).

1. Perturbation of $\Delta\psi$ for 30 sec. Following stabilization at short circuit, $\Delta\psi$ was perturbed for 30-sec periods at 30-sec intervals in the sequence $0\rightarrow\pm100$ mV (series 1*a*). The current-voltage relationship in the range -75 to 100 mV was near linear. Mean reduced values of i^a 30 sec after perturbation of $\Delta\psi$ are plotted in Fig. 1*a* and given in Table 1, column 1. On return to $\Delta\psi=0$ the final value $i^a_{0,f}$ did not differ from the original value $i^a_{0,i}\equiv1$ prior to the sequence of perturbations of $\Delta\psi$.

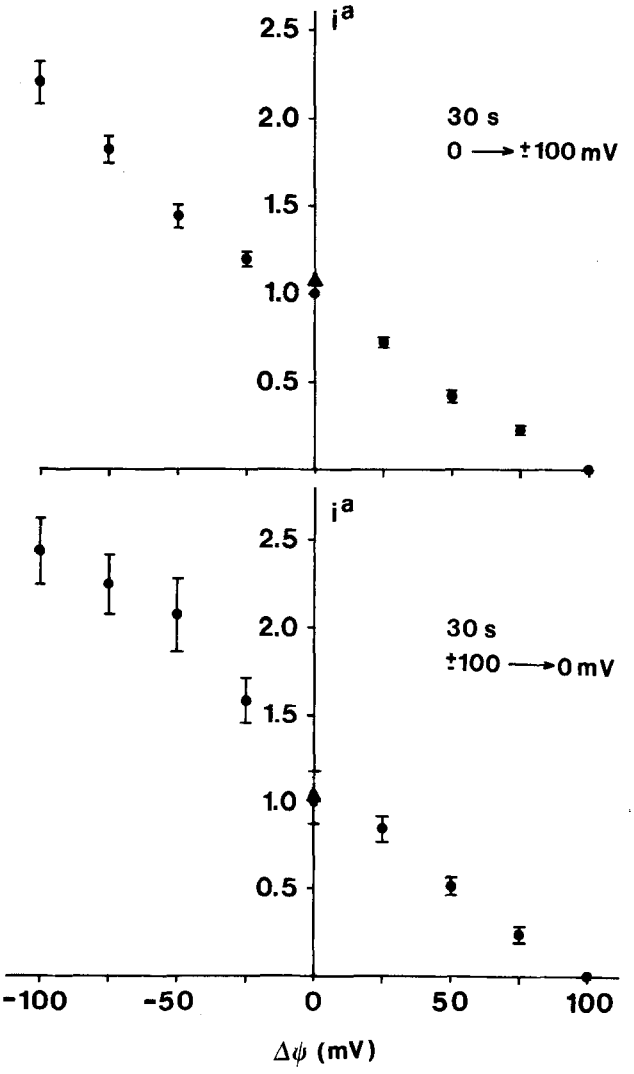


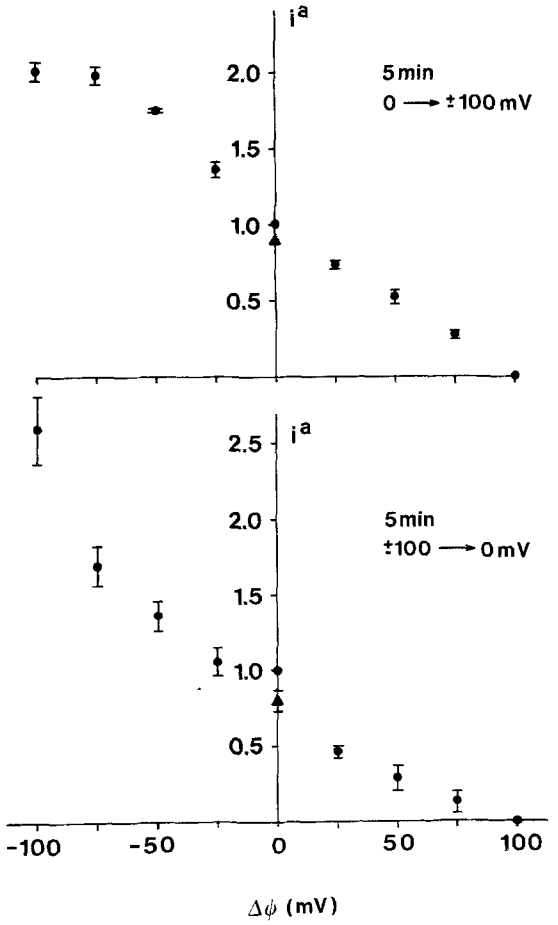
Fig. 1. Potential-dependence of the amiloride-sensitive current, expressed as the mean reduced quantity $i^a_{\Delta\psi}=(I^a_{\Delta\psi}-I^a_{100})/(I^a_0-I^a_{100})$ (*see* Methods, Eq. (2), and Results, *I*). Horizontal bars represent ±1 SE; the filled triangle represents the final value of i^a_0 following a complete sequence of perturbations of $\Delta\psi$. The upper and lower sections of the figure correspond to the "forward" and "reverse" 30-sec protocols of series 1*a* and 1*b*, respectively ($n=10$). 1*a*, reduced data ($-100\rightarrow+100$ mV): $y=1.006-0.01076$ (±0.000)x, $n=90$, $r=-0.969$, $S_{xy}=0.180$; ($-75\rightarrow+100$ mV): $y=0.9834-0.01023$ (±0.000)x, $n=80$, $r=-0.978$, $S_{xy}=0.128$; unreduced data ($-75\rightarrow+100$ mV): $y=24.00-0.2002$ (±0.027)x, $n=80$, $(\Delta\psi)_{I^a=0}=119.9$ mV

A different result was noted with perturbations in the sequence $\pm100\rightarrow0$ mV (series 1*b*) (Fig. 1*b* and Table 1, column 2). In this case there was nonlinearity for negative $\Delta\psi$. Again, however, $i^a_{0,i}$ and $i^a_{0,f}$ were not significantly different.

Table 1. Potential-dependence of reduced amiloride-sensitive current i^a for various protocols employing perturbations of $\Delta\psi$ of the indicated duration (*see* text)[a]

	1	2	3	4	5	6	7	8
Series	1*a*	1*b*	2*a*	2*b*	3*a*	3*b*	4*a*	4*b*
Duration	30 sec	30 sec	5 min	5 min	15 min	15 min	15 min	15 min
Sequence	$0\rightarrow\pm100$ mV	$\pm100\rightarrow0$ mV	$0\rightarrow\pm100$ mV	$\pm100\rightarrow0$ mV	$0\rightarrow\pm100$ mV	$\pm100\rightarrow0$ mV	$0\rightarrow\pm100$ mV	$0\rightarrow\pm100$ mV
n	10	10	5	6	11	6	4	5
i^a_{100}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
i^a_{75}	0.22 ± 0.02	0.24 ± 0.05	0.26 ± 0.03	0.13 ± 0.04	0.20 ± 0.04	0.04 ± 0.08	0.31 ± 0.06	0.30 ± 0.03
i^a_{50}	0.42 ± 0.03	0.52 ± 0.05	0.50 ± 0.04	0.29 ± 0.08	0.45 ± 0.03	0.15 ± 0.06	0.56 ± 0.05	0.56 ± 0.04
i^a_{25}	0.73 ± 0.02	0.85 ± 0.07	0.73 ± 0.03	0.46 ± 0.04	0.72 ± 0.03	0.31 ± 0.07	0.81 ± 0.03	0.81 ± 0.02
$i^a_{0,i}$	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
$i^a_{0,f}$	1.08 ± 0.09	1.03 ± 0.15	0.87 ± 0.05	0.80 ± 0.07	0.46 ± 0.04	0.53 ± 0.08	0.47 ± 0.07	0.58 ± 0.10
i^a_{-25}	1.20 ± 0.04	1.58 ± 0.13	1.35 ± 0.05	1.06 ± 0.09	1.17 ± 0.04	0.80 ± 0.08	1.26 ± 0.04	1.29 ± 0.03
i^a_{-50}	1.44 ± 0.06	2.07 ± 0.21	1.74 ± 0.01	1.37 ± 0.10	1.23 ± 0.07	1.00 ± 0.11	1.42 ± 0.11	1.46 ± 0.06
i^a_{-75}	1.82 ± 0.08	2.24 ± 0.17	1.97 ± 0.06	1.70 ± 0.13	1.18 ± 0.09	1.36 ± 0.25	1.45 ± 0.16	1.51 ± 0.09
i^a_{-100}	2.20 ± 0.12	2.43 ± 0.19	2.00 ± 0.06	2.59 ± 0.22	1.28 ± 0.13	2.00 ± 0.59	1.55 ± 0.20	1.45 ± 0.11

[a] $i^a_{\Delta\psi}=(I^a_{\Delta\psi}-I^a_{100})/(I^a_0-I^a_{100})$, where I^a_0 is the initial value of I^a at $\Delta\psi=0$. Thus i^a_0 (initial) $\equiv i^a_{0,i}\equiv1.0$, and $i^a_{100}\equiv0$; $i^a_{0,f}$ represents the final value of the reduced current at $\Delta\psi=0$ following a complete sequence of perturbations of $\Delta\psi$. (One study of series 2*a* was discarded, owing to a highly aberrant tissue response.)



2. Perturbation of $\Delta\psi$ for 5 min. In the next series of experiments $\Delta\psi$ was perturbed at 5-min intervals in the sequence $0\rightarrow\pm100$ mV (series 2*a*). Figure 2*a* and Table 1 (column 3) show the relationship between i^a and $\Delta\psi$ at the end of each 5-min period. At positive potentials i^a *vs.* $\Delta\psi$ approximates linearity, but at negative potentials there is deviation, and the difference between final and initial values of i^a_0 was significant: $\Delta=i^a_{0,f}-i^a_{0,i}=-0.13\pm0.05$ (SE) ($p(\Delta)<0.05$).

Figure 2*b* and Table 1 (column 4) show the corresponding relationship for the sequence $\pm100\rightarrow0$ mV (series 2*b*). The relationship is highly nonlinear, concave upward, and $\Delta=-0.20\pm0.07$ (SE) ($p(\Delta)<0.05$).

3. Perturbation of $\Delta\psi$ for 15 min. In an additional series of experiments $\Delta\psi$ was perturbed at 15-min intervals; series 3*a* represents the sequence $0\rightarrow\pm100$ mV. Figure 3 shows a representative study. The overshoot and prolonged relaxation of I following

Fig. 2. Potential-dependence of the amiloride-sensitive current, expressed as the mean reduced quantity $i^a_{\Delta\psi}$, as in Fig. 1. The upper and lower sections of the figure correspond to the forward and reverse 5-min protocols of series 2*a* ($n=5$) and 2*b* ($n=6$), respectively. 2*a*, reduced data ($-100\rightarrow+100$ mV): $y=1.061-0.01082\ (\pm0.000)x$, $n=45$, $r=-0.987$, $S_{xy}=0.116$; ($0\rightarrow+100$ mV): $y=0.9912-0.00987\ (\pm0.000)x$, $n=25$, $r=-0.988$, $S_{xy}=0.058$; unreduced data ($0\rightarrow+100$ mV): $y=17.48-0.1455\ (\pm0.008)x$, $n=25$, $(\Delta\psi)_{I^a=0}=120.2$ mV

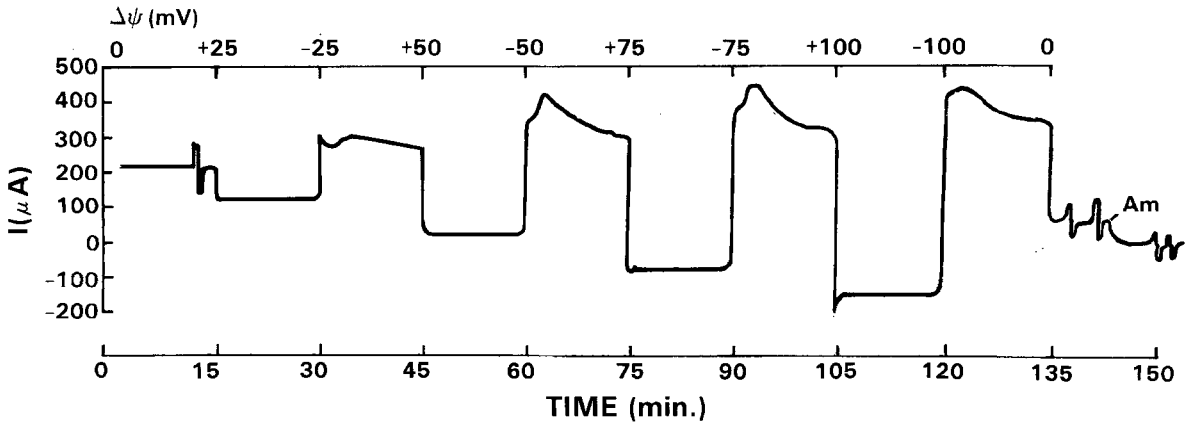


Fig. 3. Effect of transepithelial potential difference $\Delta\psi$ on current I. The arrow (Am) indicates the addition of amiloride to the mucosal medium in a concentration of 10^{-4} M

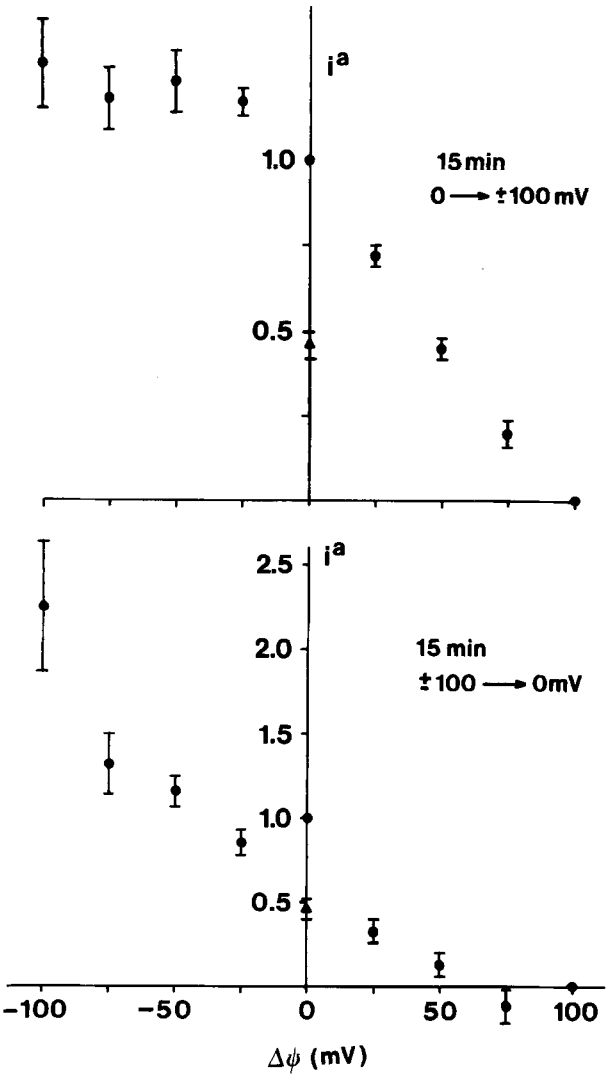


Fig. 4. Potential-dependence of the amiloride-sensitive current, expressed as the mean reduced quantitiy $i^a_{\Delta\psi}$, as in Fig. 1. The upper and lower sections of the figure correspond to the forward and reverse 15-min protocols of series 3*a* ($n=11$) and 3*b* ($n=6$), respectively. 3*a*, reduced data ($-100\rightarrow+100$ mV): $y=0.8025-0.00671\ (\pm 0.000)x$, $n=90$, $r=-0.871$, $S_{xy}=0.246$; ($0\rightarrow+100$ mV): $y=0.9773-0.01008\ (\pm 0.000)x$, $n=55$, $r=-0.973$, $S_{xy}=0.085$; unreduced data ($0\rightarrow+100$ mV): $y=26.74-0.2622\ (\pm 0.026)x$, $n=55$, $(\Delta\psi)_{I^a=0}=102.0$ mV.

clamping at large negative values of $\Delta\psi$ was observed in 7 of 11 studies; in the other 4 studies overshoot was absent or insignificant. Figure 4*a* and Table 1 (column 5) show the relationship between i^a and $\Delta\psi$ at the end of each 15-min period. Again there was impressive linearity for positive values of $\Delta\psi$, but in this instance there was a "plateau" effect for negative values of $\Delta\psi$, with i^a reaching its maximal value at about -50 mV. (Note the scale change in Fig. 4*a*.) The final value $i^a_{0,f}$ was much less than $i^a_{0,i}$, with $\Delta=-0.54\pm0.04$ (SE) ($p(\Delta)<0.001$).

Figure 4*b* and Table 1 (column 6) show the corresponding relationship for the sequence $\pm100\rightarrow0$ mV (series 3*b*). Again the curve is concave upward, as with the 5-min perturbations of Fig. 2*b*, but here the concavity is more marked, and $\Delta=-0.47\pm0.08$ (SE) ($p(\Delta)<0.001$).

In order to relate our findings to those of others, we carried out 3 experiments employing the 15 min protocol in tissues exposed to bicarbonate-free phosphate Ringer solution. Both the $0\rightarrow\pm100$ mV and $\pm100\rightarrow0$ mV sequences resulted in $I^a/\Delta\psi$ relationships closely similar to those observed with the standard bicarbonate-Ringer solution.

II. Influence of $\Delta\psi$ on κ^p

In previous studies in the toad urinary bladder it has been observed that clamping of $\Delta\psi$ at 50 mV (serosa

positive) for periods as long as 8 hr was without significant effect on the passive conductance, as judged by serosal-to-mucosal ^{22}Na flux and mucosal-to-serosal ^{36}Cl flux [42], and that clamping $\Delta\psi$ at either +50 or −50 mV for periods of 15 min had no effect on the conductance measured in the presence of 10^{-4} M mucosal amiloride [26]. Because we employed more extreme perturbations of $\Delta\psi$, it was necessary for us to examine the effects on κ^p more extensively.

In a first approach, active sodium transport was abolished by mucosal exposure to 10^{-4} M amiloride, permitting the observation of the passive current I^p while clamping $\Delta\psi$ at values unequal to zero. If prolonged perturbations of $\Delta\psi$ were without effect on κ^p, I^p should be near constant throughout the 30-min period of observation. Table 2 demonstrates, however, that for $\Delta\psi = -50$, −75, or −100 mV (serosal solution negative) I^p increased progressively; for −75 and −100 mV the effects were marked. In several tissues values of κ^p were also estimated intermittently by superimposing on the steady-state value of $\Delta\psi$ a perturbation of ±20 mV for 10 sec. These values differed insignificantly from the steady-state values, indicating that the progressive increase of I was not attributable to gradual recovery of active sodium transport. In two hemibladders in which the influence of negative potential was marked, setting $\Delta\psi = +100$ mV was without effect over a period of 30 min.

In order to test for possible interaction between active and passive pathways, a few studies were performed in the presence of normal active transport. This was conveniently done by measurements of ^{36}Cl flux, since in Dominican toad urinary bladders this flux is passive [12]. Figure 5 summarizes the results of three experiments, each employing paired hemibladders obtained from a single animal. Following baseline determinations of tracer ^{36}Cl flux for three 15-min periods at short circuit ($\Delta\psi = 0$), $\Delta\psi$ in the paired hemibladders a and b was clamped at +75 and −75 mV, respectively. Since Cl^- is transported passively, it might be expected that if κ^p were unaffected by perturbations of $\Delta\psi$ the effect of a potential of +75 mV on the influx $\vec{J}_{Cl}$ should be the same as the effect of a potential of −75 mV on the efflux $\overleftarrow{J}_{Cl}$. This was not the case. Although the initial effects of perturbation of $\Delta\psi$ on $\vec{J}$ and $\overleftarrow{J}$ were much the same, indicating close matching of paired hemibladders, during six sequential 15-min periods the efflux $\overleftarrow{J}$, measured at $\Delta\psi = -75$ mV, increased progressively, while the influx $\vec{J}$, measured at $\Delta\psi = +75$ mV, remained near constant.

Table 2. Potential-dependence of the passive current I^p [a]

$\Delta\Psi$ (mV)	n	% change in I^p		
		5 min	15 min	30 min
− 50	5	0.8±0.8	2.2±2.0	5.7± 4.4
− 75	5	4.4±1.7	8.9±2.2	17.6± 2.6
−100	11	14.0±2.6	28.4±6.6	65.2±13.0

[a] Measurements were made at the intervals indicated following mucosal exposure to 10^{-4} M amiloride. Results are reported as percent change from the value prior to administration of amiloride

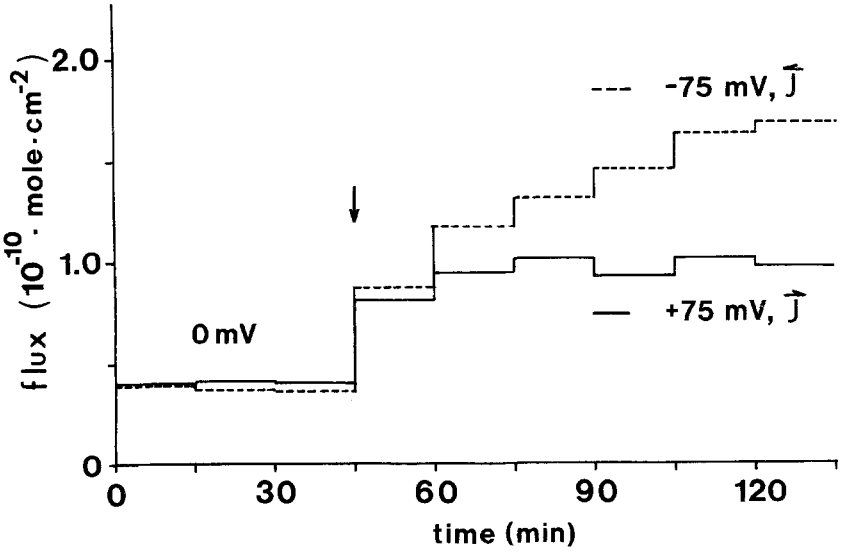


Fig. 5. Effect of potential on conductance of passive pathways, evaluated from unidirectional fluxes of tracer ^{36}Cl. Influx $\vec{J}$ and efflux $\overleftarrow{J}$ were measured in paired hemibladders a and b, respectively. Following baseline observations at short-circuit, $\Delta\psi$ was clamped at +75 mV in membrane a and at −75 mV in membrane b ($n = 3$)

III. Influence of Unstirred Layers

Since marked deviations from linearity were associated with the passage of large currents from mucosa to serosa for extended periods, the possibility was considered that the irregularities might result from incomplete mixing, with depletion of Na adjacent to the mucosal surface. This possibility was evaluated by simultaneous studies in paired membranes, employing the standard air-lift mixing system in one (series 4*a*) and a vigorous centrifugal micropump in the other (series 4*b*). In both cases $\Delta\psi$ was perturbed for 15-min intervals in the sequence $0 \rightarrow \pm 100$ mV, as in series 3*a*. At the end of each study the thickness l of the "unstirred" mucosal layer was estimated from the half-time $t_{1/2}$ for near-abolition of I_0 on exposure to 10^{-4} M amiloride, as described under Methods. The $t_{1/2}$ for the air-lift system was 7.32 ± 0.70 sec ($n=4$), and that for the pump system was 1.34 ± 0.01 sec ($n=5$), corresponding to estimated values of $1=111$ and 47.3 μm, respectively. This significant difference was unassociated with any noticeable difference in the relationship between i^a and $\Delta\psi$; the two series differed little from each other (columns 7 and 8) or from the earlier series 3*a* (column 5).

IV. Interpretation of Nonlinearity

The above results suggest that the markedly nonlinear $i^a - \Delta\psi$ relationships observed in our long protocols are not artefactual, but reflect the response of the cellular transport systems, indicating adjustments of the internal parameters. In an attempt to evaluate the effects of perturbations more precisely, we employed the classical linear equivalent circuit formulation [5, 8, 16, 24, 50, 51, 57]. According to this formulation, given a tissue maintained for an extended period at any value of $\Delta\psi$, it should be possible to evaluate κ by brief small perturbations of $\Delta\psi$ and then by the subsequent application of Eqs. (2) and (3) calculate the values of κ^a and "E_{Na}" associated with that state. (The "lumped" equivalent circuit on which such an analysis is based is shown in Fig. 6. In order to minimize the influence of the test procedure on the state of the system, $\Delta\psi$ was perturbed by ± 20 mV for only 10 sec in the determination of κ; since the positive and negative perturbations induced equivalent changes in I, κ was well defined.) This technique was applied to the study of four pairs of hemibladders utilizing the two 5-min protocols and four pairs of hemibladders utilizing the two 15-min protocols.

The results of this analysis for the two $0 \rightarrow \pm 100$ mV series are shown in Fig. 7. For "E_{Na}",

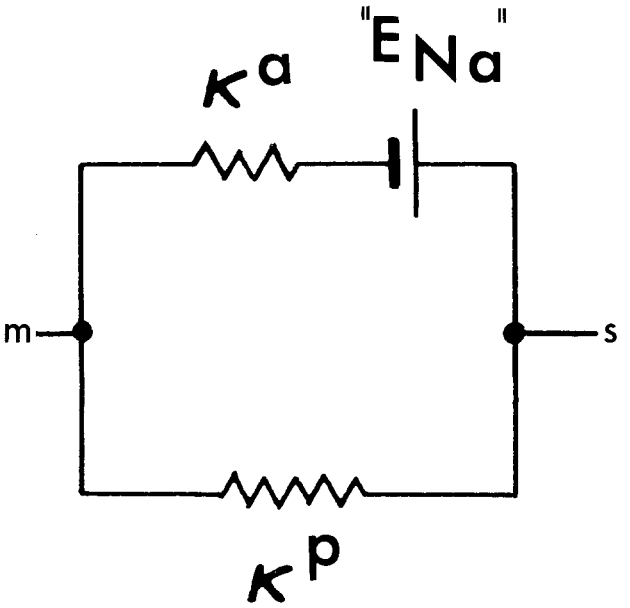


Fig. 6. Lumped equivalent circuit of transport system. In applying this model, κ^p is evaluated with the use of amiloride, and κ^a and "E_{Na}" are calculated from Eqs. (1)–(3)

except for the period at $\Delta\psi = 100$ mV, a regular relationship was observed, with negative settings of $\Delta\psi$ resulting in decrease of "E_{Na}" and positive settings resulting in increase of "E_{Na}", the magnitude of the changes increasing with the extent of deviation of $\Delta\psi$ from the equilibration value of $\Delta\psi = 0$. This result is consistent with the inverse relationship between E_{Na} and the rate of active Na transport reported by others, varying transport by perturbation of the electrical potential difference or the administration of antidiuretic hormone or amiloride [5, 16, 24][1]. (The low values of "E_{Na}" at $\Delta\psi = +100$ mV bear special comment. Since they were obtained following a period at $\Delta\psi = -75$ mV, it seemed likely that they were the result of the exposure of the tissue to these highly unphysiological conditions [3]. This conclusion is supported by the finding in each case of higher values of "E_{Na}" in the tissues of series 3*b*, which were clamped at $\Delta\psi = +100$ mV immediately following initial equilibration at short circuit. Values of "E_{Na}" in

[1] It might seem that the present results are discrepant with Feig et al.'s findings that the apparent E_{Na} decreased with decreasing concentrations of Na^+ in the mucosal medium (and thus with decreasing rates of active transport) [16]. However, as these authors point out, the apparent E_{Na} in their experiments was reduced by the chemical potential for Na^+ against which transport was occurring. When this factor is taken into account, it appears that the effects of lowering mucosal Na are consistent with those of the present study. On the other hand, Chen and Walser found linear current-voltage relationships over the range 0–150 mV in toad urinary bladder sacs, and feel that there is no need to invoke variation of E_{Na} with potential [8].

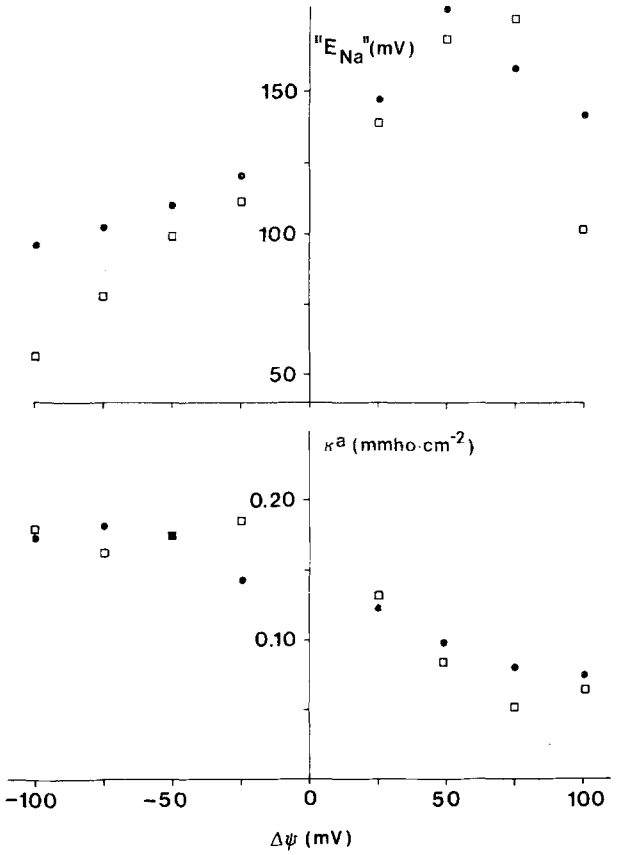


Fig. 7. Potential-dependence of parameters of lumped equivalent circuit model. At each setting of potential of the protocols of series 2*a* (5 min, 0→ ±100 mV), designated by filled circles, and series 3*a* (15 min, 0→ ±100 mV), designated by open squares, the conductance κ was measured by perturbing $\Delta\psi$ by ±20 mV for 10 sec (at 4 and 12 min, respectively), permitting the subsequent calculation of κ^a and "E_{Na}" (*see* Methods, Eqs. (1)–(3)) ($n=4$)

series 3*a* and 3*b* were 92.5±5.7 and 172.4±30.9 mV, respectively, at 4 min, and 101.3±9.2 and 168.4 ±33.1 mV, respectively, at 12 min; with $n=4$, however, the results were not demonstrably different statistically.) Also impressive was the finding that the behavior of κ^a is roughly the converse of that of "E_{Na}", with κ^a falling to low levels at positive values of $\Delta\psi$ and rising to a plateau at negative values of $\Delta\psi$.

Except for minor differences, the findings with the reverse protocols (±100→0 mV) were quite similar: "E_{Na}" varied directly and κ^a inversely with $\Delta\psi$. Again the values of "E_{Na}" at positive $\Delta\psi$ were relatively low after previous exposure of the tissue to large negative values of $\Delta\psi$.

Discussion

I. General Considerations

In attempts to characterize the kinetic and energetic factors of active transport, it is common to perturb electrical or concentration driving forces, so as to alter rates of transport and metabolism. In principle, such perturbation permits the evaluation of fundamental parameters, whether of an electrical equivalent circuit model or of a nonequilibrium thermodynamic formulation. In practice, however, it may be difficult to induce accurately measurable changes in function without also affecting the parameters which it is desired to evaluate.

Numerous workers have demonstrated that under diverse circumstances current-voltage relationships in frog skins and toad urinary bladders are highly nonlinear [e.g., 4, 9, 20, 22]. However, others have shown that with brief perturbations of $\Delta\psi$ in an appropriate range, the current-voltage relationship is approximately linear, thus defining a unique conductance κ [2, 8, 9, 44, 54]. Evaluation of the passive conductance κ^p then permits calculation of the conductance κ^a (Eq. (1)) and current I^a (Eq. (2)) presumed to represent flow of sodium in the active transport pathway, and the "electromotive force of sodium transport", "E_{Na}" (Eq. (3)). These and related techniques have often been employed to evaluate E_{Na} in the frog skin [50, 51], toad urinary bladder [3, 5, 24, 57] and other tissues [49]. (The ambiguities associated with these techniques will be considered below.)

Evaluation of the potential dependence of the rate of metabolism is more complex, since both the rate of disappearance of O_2 from bathing solutions and the rate of CO_2 appearance change more gradually than does the current following perturbation of $\Delta\psi$. Thus if J_{O_2} is evaluated from a plot of oxygen tension *vs.* time, some 2–4 min is required for the slope to reach a near-constant value following perturbation of $\Delta\psi$ [52].[2] When frog skins or toad urinary bladders are initially allowed to stabilize at short circuit and $\Delta\psi$ is then perturbed symmetrically at 6-min intervals, the dependence of J_{O_2} on $\Delta\psi$ at 4–6 min has been found to be near linear over an extensive range [28, 29, 52]. Combining these observations with concurrent linear current-voltage relationships has permitted a thermodynamic characterization of active transport [29]. Such a characterization is appropriate to the extent that the phenomenological coefficients and affinity

[2] Labarca et al. feel that a period of some 20 to 40 min is required after a change in $\Delta\psi$ in order to assure a steady state of transport and CO_2 production [26].

are well defined and near constant throughout the period of observation. Early observations in the frog skin indicated that with inappropriate protocols this is not the case, since large perturbations of $\Delta\psi$ for 15 min or longer produced a "memory effect", such that subsequent values of the short-circuit current and the associated rate of oxygen consumption differed appreciably from those noted initially [52]. The factors mediating this response can be usefully analyzed in terms of the findings of the present study.

II. Protocol Dependence of Current-Voltage Relationships

When $\Delta\psi$ was perturbed symmetrically around zero in the sequence $0 \rightarrow \pm 100$ mV at 30-sec intervals, the current-voltage relationship was near linear between -75 and $+100$ mV. With 5-min perturbations in the sequence $0 \rightarrow \pm 100$ mV near linearity was again noted, but values of i^a at negative $\Delta\psi$ lay above the projected $i^a - \Delta\psi$ plot, and the final value of the short-circuit current was slightly lower than that measured initially. These findings differ somewhat from earlier results in both frog skin [27, 36, 43, 52] and toad bladder [28, 29], where with 6-min perturbations linear relationships were observed between J^a_{Na} and/or J_{O_2} and $\Delta\psi$ over the entire range of $0 \rightarrow \pm 80$ mV. Given this variability, it may be advisable to limit future studies to the range of positive $\Delta\psi$, as suggested by Canessa et al. [5] on the basis of the findings of Bobrycki et al. [3]. Much more marked deviations were observed with the 15-min protocol. Here at negative potentials the current showed saturation, and the final values of I_0 were much lower than those measured initially.[3]

Gross irregularities were noted with the "reverse protocol" ($\pm 100 \rightarrow 0$ mV). Prolonged exposure to large negative values of $\Delta\psi$ appeared to have persistent depressant effects on transport, consistent with the impressive structural and electrophysiological effects described in both the frog skin [55] and the toad urinary bladder [3].

3 In a study of the effects of long-term perturbations of potential across the frog skin, Mandel and Curran described saturation of active Na transport similar to that observed here. With hyperpolarization, on the other hand, they observed an *S*-shaped voltage dependence [35]. The latter results are not inconsistent with ours, however, since their procedure was essentially equivalent to measuring ouabain-sensitive influx, whereas ours evaluates net flux. At large values of $\Delta\psi$, back flux through the active transport pathway can be demonstrated in the toad urinary bladder [8, 56]; influx then exceeds net flux in a manner which might account for the discrepancy between an *S*-shaped and a linear voltage-dependence.

III. Factors Contributing to Nonlinearity

In attempting to interpret the marked nonlinearities observed with prolonged perturbations of $\Delta\psi$, it was important first to consider the possibility of artifacts. One possibility was that perturbations of the magnitude and duration employed might have important effects on κ^p. Unexpectedly, in view of earlier findings of ourselves and others [26, 28, 29], we found that, although clamping $\Delta\psi > 0$ was without effect, clamping $\Delta\psi < 0$ resulted in a progressive increase in κ^p, in direct relation to both the magnitude and the duration of perturbation, both in the absence and presence of active transport (Table 2 and Fig. 5). Under circumstances where κ^p was increasing progressively with time, the use of Eq. (2), based on a falsely low estimate of κ^p, must have overevaluated I^a at negative values of $\Delta\psi$. Correction for this factor might to some extent restore linearity in Fig. 2*a* (5-min protocol), but would result in even more severe departure from linearity in the study of Fig. 3*a* (15-min protocol).

Secondly, it seemed possible that prolonged exposure to extreme negative values of $\Delta\psi$, with transport at abnormally high rates, might significantly lower the concentration of Na adjacent to the mucosal surface, while increasing the concentration at the basal lateral surface adjacent to the pump. Such concentration polarization might in principle account for subsequent abnormally low rates of transport [38, 40]. Tests for the significance of this effect at the mucosal surface were negative, but vigorous stirring of the bathing media cannot, of course, prevent accumulation of Na in the long, narrow intercellular spaces at the basolateral surface. Resultant changes in the transepithelial electrochemical potential difference for Na and thus E_{Na} would very likely be too small to account in themselves for the saturation effects observed, but elevated concentrations of NaCl might well influence membrane conductance and function [40].

IV. Interpretation of Current-Voltage Relationships; Equivalent Circuit Parameters

In an attempt to interpret effects on function more precisely, we evaluated the parameters of a standard equivalent circuit formulation. These calculations suggested that following prolonged perturbations of $\Delta\psi$ there is an inverse relationship between values of E_{Na} and the rate of transport I^a, consistent with the observations of others [5, 16]; also noted was a direct relationship between κ^a and I^a.

As discussed elsewhere, there is uncertainty as to the fundamental significance of the equivalent circuit parameters [7, 24, 33]. If, however, their meaning is tentatively accepted at face value, the behavior of "E_{Na}" appears consistent with conventional views of the regulation of active transport and metabolism [5, 55]. Thus near-linearity of the 30-sec $i^a - \Delta\psi$ relationship suggests that there is no important early effect on E_{Na}. This is reasonable since, as in an electrochemical cell of adequate capacity, the electromotive force (emf) is independent of the resistance (or potential) applied briefly at the terminals, being determined by the negative free energy (affinity) of the chemical reaction driving the current, and the kinetic factors of the system. Therefore, depending on the capacity of the system, the emf of an electrochemical cell will remain near its initial value until the rates and duration of current flow suffice to reduce the affinity significantly. The case of our biological system differs somewhat in that the affinity (presumably the negative Gibbs free energy of the reaction of ATP to form ADP and P_i) is buffered not only by the capacity of the system, but also by continuing oxidative phosphorylation, which in the steady state proceeds at a rate just adequate to compensate for the utilization of ATP to drive transport. While the stimulus for alteration in the rate of oxygen consumption is presumably alteration in the [ATP]/[ADP][P_i] ratio [37], with a highly sensitive system the alteration with brief changes of $\Delta\psi$ need not be so great as to cause appreciable change in E_{Na} [55]. With more prolonged perturbation of the rate of active transport and ATP utilization, however, the effect on levels of ATP, ADP, and P_i may no longer be adequately buffered. Thus, change over the range from hyperpolarization (at $\Delta\psi = 100$ mV) to reverse polarization ($\Delta\psi = -100$ mV) might be associated with progressive decrease of the affinity, and thus of E_{Na}. Since E_{Na} depends not only on energetic but also on kinetic factors [24], it is possible that changes in conductances may also contribute to changes in E_{Na}, but a decision on this point must await further information.

With respect to the parameter κ^a it is useful to refer to Frömter et al.'s studies in the *Necturus* urinary bladder, in which it was found that on brief perturbation of $\Delta\psi$ in the neighborhood of open circuit, the conductance of the serosal surface (κ_s) exceeded that of the mucosal surface (κ_m) by a factor of about 13 [23].[4] Presuming the applicability of these findings to our preparation, the doubling of κ^a on change of potential from $\Delta\psi = 100$ mV to $\Delta\psi = -100$ mV must have been largely attributable to the mucosal barrier, since even had κ_s become infinite it would have resulted in an increase in κ^a of less than 10 %. Further, on the basis of the specificity of the effect of amiloride on apical Na transport [19, 20] and the apparent insignificance of apical K and Cl conductance [34, 41], this large increase in κ_m must have represented an increase in $\kappa_{Na,m}$. This is consistent with Cuthbert and Shum's demonstration of an increase in amiloride binding sites with reverse polarization, presumably reflecting an increased density of Na channels [11].

The analysis above suggests that with continuing reverse polarization both the effect on Na exit ($\downarrow E_{Na}$) and that on Na entry ($\uparrow \kappa_{Na,m}$) act to sustain an increase in steady-state cell Na content. This interpretation is consistent with observations that inward current flow induces swelling of the outermost layer of the stratum granulosum in the frog skin [53] and of granular cells in the toad urinary bladder [3].

V. Detailed Analysis of Equivalent Circuit Parameters

Although the above interpretations are plausible, they depend on the assumption that the amiloride-sensitive current I^a is entirely attributable to transepithelial active sodium transport, as is implicit in the equivalent circuit model of Fig. 6. In this regard it is important to distinguish between the effects of short-term and prolonged perturbations of $\Delta\psi$. It has been repeatedly demonstrated that for Dominican toad urinary bladders mounted in chambers and studied with solutions closely similar to those of the present study, steady-state net sodium transport at short circuit is nearly equivalent to the short-circuit current [30]. We have confirmed these findings and have found also that, when J^a_{Na} is evaluated by measurement of tracer isotope fluxes, $I^a \simeq F J^a_{Na}$ during the interval 10–20 min after clamping $\Delta\psi$ at +25 or +50 mV [56]. These observations, however, provide no information as to the nature of I^a during brief perturbations of $\Delta\psi$. Although there is abundant evidence for the specificity of the effect of amiloride on sodium transport across the apical membrane [19, 20], K and Cl may contribute significantly to the current across the basal lateral membrane [34, 41]. In order to appreciate the implications of this consideration it is necessary to analyze the effects of $\Delta\psi$ and amiloride in greater detail than permitted by the lumped equivalent circuit of Fig. 6. This is done in the *Appendix*, employing the distributed equivalent circuit of Fig. 8.

[4] Frömter and co-workers determined resistances with 1–2 sec perturbations of $\Delta\psi$, whereas our determinations involved 10-sec perturbations. We do not believe that this difference is of consequence; in previous studies values of "E_{Na}" determined from values of I measured immediately or 30 sec following perturbations of $\Delta\psi$ differed insignificantly [24].

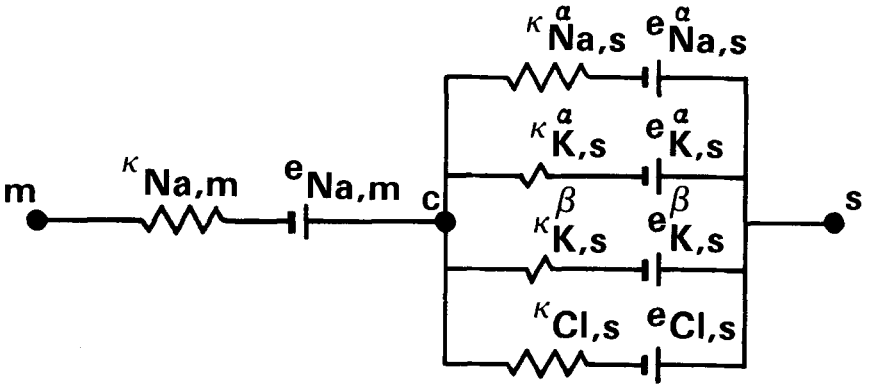


Fig. 8. Distributed equivalent circuit of cellular transport system. (For full explanation of symbols, *see* text of *Appendix*.) The superscripts α and β indicate active and passive pathways, respectively; $\kappa^{\beta}_{Na,s} \simeq 0$. We have omitted apical K and Cl currents, since they are not significant for the toad urinary bladder (Dominican Republic). For simplicity, we have also omitted the paracellular passive pathway

The main features of the analysis may be summarized as follows:

In the steady state the amiloride-sensitive current I^a represents Na flow, which is conservative (i.e., the rate of apical Na entry equals the rate of basal lateral Na exit). Hence the "force" promoting Na exit from the cell ($e^a_{Na,s} - \Delta\psi_s$) is related to the force promoting entry ($e_{Na,m} - \Delta\psi_m$) in the ratio $\kappa_{Na,m}/\kappa_{Na,s}$, where $\kappa_{Na,m}$ and $\kappa_{Na,s}$ are the partial Na conductance at the mucosal and serosal cell surface, respectively. If now the transmembrane electrical potential difference $\Delta\psi \equiv \Delta\psi_m + \Delta\psi_s$ is perturbed to a new value $\Delta\psi' = \Delta\psi + \delta\Delta\psi \equiv (\Delta\psi_m + \delta\Delta\psi_m) + (\Delta\psi_s + \delta\Delta\psi_s)$, after a very brief transient $\delta\Delta\psi_s/\delta\Delta\psi_m = \kappa_m/\kappa_s$, where κ_m and κ_s represent the *total* conductance at the m and s surface, respectively. The effect of such a perturbation on cell ion content will depend on the pattern of partial ionic conductances at the opposite plasma membranes. Since in general $\kappa_{Na,m}/\kappa_{Na,s} \neq \kappa_m/\kappa_s$, it would be expected that for some period following perturbation of $\Delta\psi$ Na flow will be nonconservative (i.e., mucosal and serosal Na fluxes will differ), resulting in change in cell Na (and other ion) content. The rate of change of cell ion concentrations will depend on the magnitude of the discrepancy between mucosal and serosal fluxes, cell volume, and the rate of osmotic adjustments. With the passage of sufficient time the system will relax to a true steady state, in which all nonconservative flows will have vanished, so that again ion concentrations will be invariant with time and I^a will closely approximate the transepithelial Na current.

Given this background we can now enquire into the significance of the values of κ^a and "E_{Na}" determined by various means based on measurement of electrical currents and "10 second" conductances. Since on brief perturbations of $\Delta\psi$, I^a cannot be assumed to represent net transepithelial Na transport, the amiloride-sensitive conductance κ^a calculated from Eq. (1) is not precisely equivalent to the Na conductance of the active transport pathway, κ^a_{Na} (*see Appendix*, Eq. (A23)). Accordingly, while the lumped circuit "E_{Na}" calculated from Eqs. (2) and (3) will evaluate the transepithelial potential difference necessary to reduce the total cellular current to zero (to within the accuracy of the linear formulation), it may not be assumed to evaluate precisely the true value of E_{Na}, i.e., the value of $\Delta\psi$ adequate to reduce basal lateral active Na transport to zero. (*See* Eqs. (A10) and (A21).) Similar reservations apply also to other methods depending on the evaluation of conductance from a brief pulse, e.g., the widely used method of Yonath and Civan [57]. (We shall call the E_{Na} evaluated in this manner $(E_{Na})_{Y-C}$.)

Although information which would permit precise evaluation of κ^a_{Na} and thus E_{Na} is lacking, it is of interest to consider the relationships which would obtain under possible limiting conditions. If, on the one hand, $\kappa_{Na,s} \gg \kappa_{Na,m}$ (and thus $\kappa_s > \kappa_{Na,s} \gg \kappa_{Na,m}$), κ^a_{Na} does not deviate sufficiently from κ^a to invalidate the perturbation techniques for evaluation of E_{Na} since, as is seen from Eq. (A23), $\kappa^a_{Na}/\kappa^a \simeq 1$, and from Eqs. (A21) and (A25), "E_{Na}" $\simeq (E_{Na})_{Y-C} \simeq E_{Na}$.[5] In this case our experimental results would indicate that E_{Na} varies directly with $\Delta\psi$ as discussed. If, on the other hand, $\kappa_{Na,s}$ is not $\gg \kappa_{Na,m}$, Eq. (A23) shows that $\kappa^a_{Na} < \kappa^a$. In this case, Eq. (A21) predicts a direct relationship between "E_{Na}" and $\Delta\psi$, as observed in the present study, even if E_{Na} is unaffected by variation of $\Delta\psi$. Also, it would not be possible to

[5] In their serosal impalements of *Necturus* urinary bladders with widely varying rates of transport, Frömter et al. found that $\Delta\psi_s$ was near constant at about 90 mV, whereas $\Delta\psi_m$ varied from some -60 to $+50$ mV [23, Fig. 9]. This raises the possibility that $(e^{\alpha}_{Na,s} - \Delta\psi_s) \ll (e_{Na,m} - \Delta\psi_m)$, and $\kappa_{Na,s} \gg \kappa_{Na,m}$, so that small changes in $\Delta\psi_s$ resulting from changes in $\kappa_{Na,m}$ may result in large changes in the rate of transepithelial transport. Frömter et al. have suggested, on the other hand, that their findings might be due to an increased number of operating transport units, since the transition from low to high transport rates was associated with a decrease of the serosal membrane resistance. In the rabbit urinary bladder, however, both the serosal membrane potential and the serosal resistance are reported to be near constant [31]. Schultz et al. have reported that in the rabbit colon R^s_{Na}/R^m_{Na} $(\equiv \kappa_{Na,m}/\kappa_{Na,s}) = 100/1570 \simeq 0.06$ [45].

evaluate E_{Na} precisely by combined measurements of current and "short-term" conductances, as attempted by ourselves and others. Thus, whereas κ^a varies, κ^a_{Na} and E_{Na} may be near constant following perturbations of $\Delta\psi$ of moderate magnitude and duration, permitting the evaluation of E_{Na} from the steady-state intercept $(\Delta\psi)_{I^a=0}$. (For the linear regions of the $I^a - \Delta\psi$ plots of series $2a$ (5 min) and $3a$ (15 min), these values were 120.2 and 102.0 mV, respectively.)

Another possibility, of course, is that κ^a_{Na}/κ^a may vary with both time and $\Delta\psi$ following a perturbation. Clearly, detailed electrophysiological studies of both apical and basal lateral plasma membrane function are required in order to choose among these and still other alternatives [21, 31, 33].

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Appendix

Equivalent Circuit: Steady State and Transient Relationships

General Considerations

In the analysis of transepithelial active sodium transport by means of electrical equivalent circuit models it is common to consider only Na flow and to "lump" the electrical properties of the apical ("mucosal") and basal-lateral ("serosal") membranes [5, 8, 16, 24, 50, 51, 57] (Fig. 6). However, in order to deal with the various data above, it is necessary to consider a "distributed" equivalent circuit incorporating the contribution of each significant ion flow at each rate-limiting barrier. For this purpose we follow the treatments of Schultz, Frizzell and Nellans [46], Lindemann [32], and Hviid Larsen and Kristensen [25] (Fig. 8). Unlike the latter authors, however, we postulate that the rate of the pump is dependent on the total electrochemical potential difference for Na at the serosal surface, rather than only the cellular Na concentration, since their assumption would require that in the absence of active transport the pump would maintain an infinite chemical potential difference for Na. As discussed by Schultz et al., there appears to be no need to invoke stoichiometry between active Na and K transport at the serosal surface. Assuming significant electrical coupling between cells [39] and the absence of important cytoplasmic electrical potential gradients, we assign a unique intracellular electrical potential ψ_c. It should be noted, however, that although the individual circuits join at point c, the assignment of an electromotive force for each ion at each surface, in addition to taking account of different concentration profiles and transport mechanisms, assures that each ionic current is discrete. Thus the formulation deals with Schultz et al.'s objections [46] to Cuthbert's model [10]. It is appreciated, however, that the partial conductances and electromotive forces (emf's) at each surface are lumped parameters, possibly incorporating different contributions from different cell types. In addition, of course, the analysis suffers from the limitations common to simple electrical circuit models in assuming linearity of the currents in the electrical and chemical potential differences. (For nonlinear models, *see* [21] and [32].)

A general analysis of function would require the explicit consideration of all partial conductances and emf's at the two surfaces. However, for the tissue under study here it is possible to ignore certain of the conductances. Thus, at the mucosal surface,

$$\kappa_m = \kappa_{Na,m} + \kappa_{K,m} + \kappa_{Cl,m} \simeq \kappa_{Na,m}. \tag{A1}$$

We have considered $\kappa_{K,m} \simeq 0$ since despite the presence of a K pump at the serosal surface there is no significant active transepithelial K transport in the Dominican Republic toad urinary bladder [41]. Also, Macknight's studies indicate that $\kappa_{Cl,m} \simeq 0$ and that there is no significant NaCl co-transport at the apical surface [34]. At the serosal surface,

$$\begin{aligned}\kappa_s &= \kappa_{Na,s} + \kappa_{K,s} + \kappa_{Cl,s} \\ &= \kappa^{\alpha}_{Na,s} + \kappa^{\beta}_{Na,s} + \kappa^{\alpha}_{K,s} + \kappa^{\beta}_{K,s} + \kappa_{Cl,s}\end{aligned} \tag{A2}$$

where the superscripts α and β refer to active and passive pathways, respectively. Potassium transport at the serosal surface is considered to be by means of a parallel pump-leak system. (If justified by further information, it is straightforward to discriminate between basolateral active and passive Cl pathways as well.) Although Na flow through the pump pathway is reversible [8, 56], Canessa et al. have shown that recycling by way of a parallel serosal leak is not demonstrable [6]; hence we set $\kappa^{\beta}_{Na,s} = 0$ and $\kappa^{\alpha}_{Na,s} = \kappa_{Na,s}$.

The "forces" at the two surfaces sum to give the total transepithelial force. Thus, for electrical potential.

$$\Delta\psi_m + \Delta\psi_s = \Delta\psi. \tag{A3}$$

We use the symbol $e_{i,m(s)}$ to represent the combined contributions of the negative chemical potential dif-

ference ("Nernst potential"), $-(RT/z_iF)\,\Delta \ln c_i$, and any active transport mechanism which may be operative. Then, with the use of identical Na-Ringer solutions at each surface, we have for Na, with a pump of emf E_{Na} at the serosal surface,

$$e_{Na,m}+e^{\alpha}_{Na,s}=E_{Na}. \tag{A4}$$

For K, with a "pump" (α) and "leak" (β) at the serosal surface,

$$e_{K,m}+e^{\alpha}_{K,s}=-E_K \tag{A5}$$

$$e_{K,m}+e^{\beta}_{K,s}=0. \tag{A6}$$

For Cl, whose transepithelial transport is considered to be entirely passive in the toad urinary bladder (Dominican Republic),

$$e_{Cl,m}+e_{Cl,s}=0. \tag{A7}$$

Steady-State Relationships

In the steady state, the transcellular current is exclusively attributable to Na:

$$I^a_{Na}=\kappa_{Na,m}(e_{Na,m}-\Delta\psi_m)=\kappa_{Na,s}(e^{\alpha}_{Na,s}-\Delta\psi_s). \tag{A8}$$

Introducing now for the total cellular conductance of Na the symbol

$$\kappa^a_{Na}\equiv\frac{\kappa_{Na,m}\kappa_{Na,s}}{\kappa_{Na,m}+\kappa_{Na,s}} \tag{A9}$$

and combining Eqs. (A3), (A4), and (A8) gives the appropriate steady-state relationship between the conductance and emf of the lumped equivalent circuit model for the active Na transport pathways:

$$I^a_{Na}=\kappa^a_{Na}(E_{Na}-\Delta\psi). \tag{A10}$$

For K, although there is no transepithelial current, there are flows to be considered at the serosal surface. These are given by

$$I_K=I^{\alpha}_{K,s}+I^{\beta}_{K,s}=\kappa^{\alpha}_{K,s}(e^{\alpha}_{K,s}-\Delta\psi_s)+\kappa^{\beta}_{K,s}(e^{\beta}_{K,s}-\Delta\psi_s)=0 \tag{A11}$$

Introducing (A5) and (A6),

$$I^{\alpha}_K=-I^{\beta}_K=-\frac{\kappa^{\alpha}_{K,s}\kappa^{\beta}_{K,s}}{\kappa^{\alpha}_{K,s}+\kappa^{\beta}_{K,s}}E_K. \tag{A12}$$

As emphasized by Schultz et al., the above formulations are applicable only when the system is in a steady state in which "ionic compositions and transepithelial ionic movements are time-independent; the underlying assumptions preclude the application of these considerations to transient states." In this regard, however, it is important to distinguish between the system as a whole, and the rate-limiting barriers at the cell surfaces. Since the volumes of these different regions differ by orders of magnitude it is to be anticipated that following a perturbation of $\Delta\psi$ the plasma membranes will reach new quasi-steady states long before the cell reaches a true steady state. That is to say, promptly following a perturbation each ionic flow across each plasma membrane becomes near conservative, although differing at the two cell surfaces. Accordingly, plasma membrane concentrations and electrical parameters assume values appropriate for the simultaneous values in the contiguous bathing solution and cell interior, thereafter changing *pari passu* with the parameters of the cytoplasm. These considerations permit the analysis of the function of the epithelial system even during transient periods when cell parameters are slowly changing.

Transient Relationships: Effects of Perturbation of $\Delta\psi$ on Cell Composition

On perturbing $\Delta\psi$ to a new value $\Delta\psi'=\Delta\psi+\delta\Delta\psi=(\Delta\psi_m+\delta\Delta\psi_m)+(\Delta\psi_s+\delta\Delta\psi_s)$, following a very brief period attributable to electrical capacitance of the tissue, the electrical current across the cells becomes conservative. Therefore,

$$\delta I^a=-\kappa_m\delta\Delta\psi_m=-\kappa_s\delta\Delta\psi_s,$$

so that, with Eq. (A3),

$$\delta\Delta\psi_m=\frac{\kappa_s\delta\Delta\psi}{\kappa_m+\kappa_s};\qquad \delta\Delta\psi_s=\frac{\kappa_m\delta\Delta\psi}{\kappa_m+\kappa_s} \tag{A13}$$

$$\delta I^a=\delta I_m=\delta I_s=-\left(\frac{\kappa_m\kappa_s}{\kappa_m+\kappa_s}\right)\delta\Delta\psi. \tag{A14}$$

(These relations apply with perturbations long enough to assure conservation of current flow across the epithelium, but brief enough to avoid significant changes in κ_m and κ_s.)

Perturbations of $\Delta\psi$ will alter the ionic currents at each membrane. Thus, for Na at m, shortly after perturbation of $\Delta\psi$

$$\delta I_{Na}=\kappa_{Na,m}(-\delta\Delta\psi_m)=-\frac{\kappa_{Na,m}\kappa_s}{\kappa_m+\kappa_s}\delta\Delta\psi. \tag{A15}$$

(The validity of these relationships depends on the fact that the mucosal plasma membrane is in a quasi-steady state. Hence the Na currents entering and leaving the mucosal membrane are sensibly identical, so that $I_{Na,m}$ is conservative and hence well defined.) Similarly, at s,

$$\delta I_{\mathrm{Na},s}=\kappa_{\mathrm{Na},s}(-\delta\Delta\psi_s)=-\frac{\kappa_{\mathrm{Na},s}\kappa_m}{\kappa_m+\kappa_s}\delta\Delta\psi. \quad (A16)$$

It is seen that transcellular Na flow is conservative (i.e., $\delta I_{\mathrm{Na},m}=\delta I_{\mathrm{Na},s}$) if and only if $(\kappa_{\mathrm{Na},m}/\kappa_{\mathrm{Na},s})=(\kappa_m/\kappa_s)$. In this case perturbation of $\Delta\psi$ will not alter cell Na. Since in the present case, however, $(\kappa_{\mathrm{Na},m}/\kappa_{\mathrm{Na},s})>(\kappa_{\mathrm{Na},m}/\kappa_s)=(\kappa_m/\kappa_s)$, it is seen that with negative perturbation of $\Delta\psi$ (as from open circuit to short circuit or reverse polarization) $\delta I_{\mathrm{Na},m}>\delta I_{\mathrm{Na},s}$, so that cell Na will increase. For K and Cl the case is different. Since $\kappa_{\mathrm{K},m}$ and $\kappa_{\mathrm{Cl},m}\simeq 0$, the effect of a negative perturbation of $\Delta\psi$ must be to decrease cell K and increase cell Cl, as is seen from Eqs. (A2), (A11) and (A13):

$$\delta I_{\mathrm{K},s}=-\frac{\kappa_{\mathrm{K},s}\kappa_m\delta\Delta\psi}{\kappa_m+\kappa_s};\quad \delta I_{\mathrm{Cl},s}=-\frac{\kappa_{\mathrm{Cl},s}\kappa_m\delta\Delta\psi}{\kappa_m+\kappa_s}. \quad (A17)$$

Maintenance of macroscopic electroneutrality requires that $\delta I_{\mathrm{Na},m}-\delta I_{\mathrm{Na},s}=\delta I_{\mathrm{K},s}+\delta I_{\mathrm{Cl},s}$. The extent to which increase in cell Na will be compensated by decrease in K rather than increase in Cl will be determined by the relative magnitudes of $\kappa_{\mathrm{K},s}$ and $\kappa_{\mathrm{Cl},s}$; it is to be expected that these effects will influence the changes in κ^a observed following long-term perturbations of $\Delta\psi$. With the passage of time the intracellular potential profile, emf's, and/or conductances will relax to steady-state values such that cellular I_{Na} is again conservative and I_{K} and $I_{\mathrm{Cl}}=0$, as is consistent with the equivalence of amiloride-sensitive current and net transcellular Na flux demonstrated following $\geqq$10-min perturbations of $\Delta\psi$ [56].

Effects of Amiloride (at Fully Inhibitory Concentrations)

Assuming, as is widely accepted, that amiloride acts by preventing passive Na flux across the mucosal surface, Eqs. (A1) and (A9) give

$$\kappa_m^A=\kappa_{\mathrm{Na},m}^A=(\kappa_{\mathrm{Na}}^a)^A=0 \quad (A18)$$

so that perturbation of the potential in the presence of amiloride gives the passive conductance κ^p, attributable to paracellular pathways. In the steady state, Eq. (A8) shows that

$$\Delta\psi_s^A=(e_{\mathrm{Na},s}^{\alpha})^A. \quad (A19)$$

It can be seen from Eqs. (A13) and (A18) that on perturbing $\Delta\psi$ in the presence of fully inhibitory levels of amiloride

$$\delta\Delta\psi_m^A=\delta\Delta\psi;\quad \delta\Delta\psi_s^A=0. \quad (A20)$$

(These relationships depend critically on the evidence that in the toad urinary bladder (Dominican Republic) $\kappa_{\mathrm{K},m}$ and $\kappa_{\mathrm{Cl},m}\simeq 0$.)

Evaluation of Equivalent Circuit Parameters

Because of the specificity of the effect of amiloride on Na flux across the mucosal surface, we and others have employed this agent to evaluate the parameters of the lumped equivalent circuit of the Na active transport system [5, 24, 46]. This has involved evaluation of the conductance of the active Na transport pathway by brief perturbation of $\Delta\psi$ in the absence and presence of amiloride, then applying Eq. (1) to evaluate κ^a and Eq. (3) to evaluate "E_{Na}". It should be noted that this calculation combines values of I measured in a well established steady state with values measured several seconds after perturbation of $\Delta\psi$. Accordingly, the value "E_{Na}" derived from Eq. (3) will be a reliable estimate of E_{Na} only to the extent that κ^a provides an accurate value of κ_{Na}^a, as is shown by combination of Eqs. (3) and (A10):

$$\text{“}E_{\mathrm{Na}}\text{”}=\left(\frac{\kappa_{\mathrm{Na}}^a}{\kappa^a}\right)E_{\mathrm{Na}}+\left(1-\frac{\kappa_{\mathrm{Na}}^a}{\kappa^a}\right)\Delta\psi. \quad (A21)$$

The present analysis indicates, however, that κ^a is not precisely equal to κ_{Na}^a. Combining Eq. (1) with Eqs. (A1) and (A14) shows that κ^a incorporates conductances in addition to those of the Na active transport pathway:

$$\kappa^a=\frac{\kappa_{\mathrm{Na},m}}{1+\kappa_{\mathrm{Na},m}/\kappa_s} \quad (A22)$$

giving with Eq. (A9)

$$\frac{\kappa_{\mathrm{Na}}^a}{\kappa^a}=\frac{1+\kappa_{\mathrm{Na},m}/\kappa_s}{1+\kappa_{\mathrm{Na},m}/\kappa_{\mathrm{Na},s}}. \quad (A23)$$

Also of interest is the accuracy of the evaluation of E_{Na} by the method of Yonath and Civan [57]. This method depends on the assumption that antidiuretic hormone (ADH) enhances active Na transport by increasing the Na conductance of the mucosal membrane, without affecting E_{Na} or the conductance of parallel passive pathways. It is then considered that a plot of total conductance κ (evaluated from 10-sec pulses) against the short-circuit current I_0 will be linear, with a slope $d\kappa/dI_0$ inversely proportional to E_{Na}. (We shall call the E_{Na} evaluated in this manner $(E_{\mathrm{Na}})_{\mathrm{Y-C}}$.) Consideration of Eq. (A10) shows that the true value of E_{Na} is given by $(dI_{\mathrm{Na}}^a/d\kappa_{\mathrm{Na}}^a)_{\Delta\psi=0}\equiv(dI_0/d\kappa_{\mathrm{Na}}^a)$. Thus

$$(E_{\mathrm{Na}})_{\mathrm{Y-C}}=(d\kappa_{\mathrm{Na}}^a/d\kappa)\,E_{\mathrm{Na}}. \quad (A24)$$

Assuming that ADH acts only on $\kappa_{Na,m}$, $d\kappa^a_{Na}/d\kappa \equiv (d\kappa^a_{Na}/d\kappa_{Na,m})/(d\kappa/d\kappa_{Na,m})$. The numerator is readily evaluated by differentiating the expression of Eq. (A9) with respect to $\kappa_{Na,m}$. The denominator is obtained by introducing Eq. (A1) into (A14) and differentiating $(\delta I/\delta \Delta\psi)$ with respect to $\kappa_{Na,m}$. Thus

$$(E_{Na})_{Y-C} = \left[\frac{1+\kappa_{Na,m}/\kappa_s}{1+\kappa_{Na,m}/\kappa_{Na,s}}\right]^2 E_{Na} = \left[\frac{\kappa^a_{Na}}{\kappa^a}\right]^2 E_{Na}. \quad (A25)$$

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