Ouabain on Active Transepithelial Sodium Transport in Frog Skin

Studies with Microelectrodes

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ABSTRACT Studies were done with isolated frog skin to determine the effects of 10^{-4} M ouabain on the electrophysiological parameters of outer and inner barriers of the Na-transporting cells. Microelectrodes were used to impale the skins from the outer surface to determine the intracellular voltages (V_o) under conditions of short-circuiting and under conditions where a voltage clamp was used to vary the transepithelial voltage, V_T. From this, the electrical resistances of outer (R_{So}) and inner (R_{Si}) barriers were estimated. In addition, the driving force for active transepithelial Na transport (E_{iNa} = E_{iI}) was estimated from the values of V_T when the V_o = 0 mV (Helman and Fisher, 1977. J. Gen. Physiol. 69: 571-604). Studies were done with skins bathed with the usual 2.4 meq/liter [K], in the inner solution as well as with reduced [K]_i of 0.5 and 0 meq/liter. Characteristically, the responses to ouabain could be described by an initial rapid phase (5-10 min) during which time the R_{Si} was increased markedly and the E_{iI} was decreased from control values. Thereafter, during the slow phases of the response, the resistances of both outer and inner barriers increased continuously and markedly with time leading ultimately to essentially complete inhibition of the short-circuit current. Similar studies were done with skins exposed to 10^{-4} M amiloride in the outer solution. Although estimates of R_{Si} could not be obtained under these conditions, the effects on the V_o and E_{iI} were similar to those observed for the Na-transporting skins. However, the magnitudes of the effects were less and relatively slower than observed for the Na-transporting skins. The results of these studies were analyzed within the context of a proposed electrical model that takes into account the observation that the magnitude of the voltage at the inner barrier appears to exceed the equilibrium potential for K especially when transepithelial Na transport is inhibited at the apical barrier of the cells.

INTRODUCTION

Of the inhibitors of active Na transport by epithelial tissues, the action of the cardiac glycoside, ouabain, is of particular interest because of its specificity of binding to the (Na + K)-ATPase at the basolateral borders of the epithelial cells (Farquhar and Palade, 1966; Ernst, 1972; Stirling, 1972; Mills et al., 1977). In...
frog skin and other epithelia ouabain causes inhibition of transepithelial Na transport as measured either isotopically or electrically as the short-circuit current, $I_{sc}$ (Koefoed-Johnsen, 1957; Herrera, 1966; Solinger et al., 1968; Nagel and Dörge, 1971; Robinson and Macknight, 1976). In order to explain ultimately the inhibition by ouabain of the $I_{sc}$, it will be necessary to understand the relationship between the forces and ionic flows occurring at apical and basolateral barriers of the Na transporting cells. In this regard, the present studies were concerned primarily with an evaluation of the electrical changes occurring at these barriers as evaluated with intracellular voltage recording microelectrode techniques.

Preliminary reports of this work have appeared elsewhere (Helman and Nagel, 1977; Nagel and Helman, 1977a; Nagel and Helman, 1977b).

MATERIALS AND METHODS

Microelectrode studies were done with belly skins of *R. pipiens berliendieri* (Mogul Ed, Oshkosh, Wis.; Southwestern Scientific Co., Tucson, Ariz.) with methods described in detail elsewhere (Nagel, 1976; Helman and Fisher, 1977).

In general, the skins were short-circuited continuously with a voltage clamp and the short-circuit current allowed to stabilize for 1-3 h. The skins were bathed symmetrically with a Cl-$\text{HCO}_3$ Ringer (microelectrode studies) containing in millimolar: 100.0 NaCl, 2.4 KHCO$_3$, 2.0 CaCl$_2$, and 11.1 glucose or in a sulfate Ringer (studies of the $I_{sc}$-$V_{sc}$ relationships) containing in millimolar: 57.2 Na$_2$SO$_4$, 2.4 KHCO$_3$, and 1.2 CaSO$_4$. In some studies the K concentration of the inner bathing solution was decreased from the usual 2.4 to either 0.5 and 0.0 mM by substitution of K with Na. These solutions will be referred to by the notation 2.4 [K], 0.5 [K], and 0 [K]. Ouabain (Sigma Chemical Co., St. Louis, Mo.) was added to the inner solution at a concentration of $10^{-4}$ M except in those studies of the $I_{sc}$-$V_{sc}$ relationships where the amiloride concentration was $5 \times 10^{-7}$ M. In studies of the $I_{sc}$-$V_{sc}$ relationships, skins were mounted in chambers described previously in detail (Helman and Miller, 1971).

Chambers

In microelectrode studies, skins were mounted horizontally in chambers which were constructed to permit the solution bathing the inner surface of the skin to be changed without disturbing the position of the tip of the microelectrode. The chamber system described by Nagel (1976) and the system with essentially no hydrostatic pressure across the skin described by Helman and Fisher (1977) were used. The latter system was modified for later studies to include a stainless steel grid support at the corial side of the skin, and this permitted a subpressure of ~30 cm H$_2$O to be applied to hold the skin against the support and to allow continuous flow of fluid through the inner chamber throughout the entire period of study. The flow rate was usually 3-6 ml/min through a chamber volume of ~0.3 ml. Owing to the dead space between the fluid reservoir and the inner chamber, a delay of usually 30-60 s and in some cases longer (flow rates <3 ml/min) occurred before ouabain added to the reservoir reached the chamber. It should be noted that the results of studies done in the presence or absence of a hydrostatic pressure gradient and in the presence or absence of a grid were the same.

Microelectrodes

Microelectrodes were made with a horizontal puller (Narishige Scientific Instrument Lab., Ltd., Tokyo, Japan) using Omega dot glass capillaries (Frederick Haer & Co.,
Brunswick, Maine, 30-30-1) immediately before use. The tips of the electrodes were filled with 3 M KCl by capillarity and thereafter backfilled. Tip resistances ranged between 15 and 40 MΩ. In order to rule out systematic effects of KCl leakage from the tips into the cells during impalement, some tips were filled with 150 mM KCl, and the values of intracellular voltage were compared with those observed with 3 M KCl-filled electrodes. Since the values of intracellular voltage were essentially the same, all studies were done with 3 M KCl-filled electrodes. Additional studies were done with electrodes filled with either 4 M K-acetate or 3 M NH₄NO₃, and here, too, the intracellular voltages were similar. We attempted to determine the rate of K leakage using electrodes filled with 3 M KCl and the highest concentration of ⁴²K available to us (20–40 mCi/ml ⁴²K, New England Nuclear, Boston). In most electrodes the rate of K leakage was so low that an estimate of leakage rate could not be obtained despite the high level of ⁴²K activity in the pipettes. It should be noted also that the intracellular voltages remain essentially constant for considerable periods of time consistent with the belief that K leakage from the tips is negligible and at best unimportant. Moreover, reimpalement of the skin at any time during an experiment gave instantaneous voltages not different from those observed with microelectrodes used to record chronically.

Method of Procedure

MICROELECTRODE STUDIES The skins were impaled from above in a perpendicular direction with the microelectrode advanced from the outer solution. A voltage clamp was used in two ways: first, to maintain the skins at a transepithelial voltage of zero thereby permitting the short-circuit current, Iₛₑ, to be monitored continuously. The values of Vₒ for skins short-circuited will be referred to with the symbol Vₒᵣ and are identical in magnitude to those of the inner barrier, Vᵢ, when Vᵣ = 0. Second, to determine the values of Eᵣ that are thought also to provide estimates of E Na, the voltage clamp was used to vary the Vᵣ (pulse duration = 600 ms) in order to determine the value of Vᵣ that brings the Vₒ to zero millivolts. This follows the idea proposed before that the E Na can be estimated from those values of Vᵣ = Eᵣ at which transepithelial Na transport and the Vₒ are thought to be not different from zero (Helman and Fisher, 1977).

From the slope of the relationship between Vᵣ and Vₒ, the fractional transcellular resistance of the outer barrier, %Rₒᵣ, was estimated as described in detail elsewhere (Helman and Fisher, 1977):

\[
\% Rₒᵣ = \frac{Vₒᵣ}{Eᵣ} \times 100 = \frac{\Delta Vₒ}{\Delta Vᵣ} \times 100 = \frac{Rₒᵣ}{(Rₒᵣ + Rᵣ)} \times 100. \tag{1}
\]

The symbols Rₒᵣ and Rᵣ refer to the specific resistances of the outer and inner barriers, respectively. It should be noted that the Rₒᵣ is determined when the Vₒ is negative. No attempt was made in the present studies to evaluate the Rᵣ when Vₒ is positive (see Helman and Fisher, 1977).

Since

\[
Rₒᵣ + Rᵣ = Rₒ = Eᵣ/Iₛₑ, \tag{2}
\]

the specific resistances in ohm-cm² could be estimated.

After the skins had been allowed to stabilize while short-circuited, the control values of Vₒᵣ were determined. It was not unusual to obtain an impalement in which the values of Vₒᵣ, %Rₒᵣ, and Eᵣ would remain stable for considerable periods of time often exceeding 1 h or more. Thus, we could measure from a single impalement control values for 10–20 min and then follow the time-course of the changes caused by ouabain (10⁻⁴ M) added to
the inner solution. The control values of $V_{m}$ observed among cells of a single skin were remarkably similar and in preliminary studies (not reported) a number of cells were punctured at intervals of 5–10 min throughout control and experimental periods. Despite a slightly larger scatter of the data, these studies gave the same results as obtained by continuous monitoring from a single impalement.

$I_{\tau}-V_{\tau}$ Relationships Skins were short-circuited continuously, and the $I_{\tau}-V_{\tau}$ relationships determined during control and ouabain experimental periods with methods described in detail elsewhere (see for example Helman and Fisher, 1977). From the steady-state values of transepithelial current and voltage, $I_{\tau}$ and $V_{\tau}$, respectively, the slope resistances were calculated with linear regression analysis and the transepithelial voltage $E_{1}$ defined at the intersection of slope resistances $R_{a}$ and $R_{b}$ (see Fig. 1). Previous studies indicated that the values of $E_{1}$ were the same as those of the $E_{Na}$ of Ussing and Zerahn (1951) and the same as those of $E_{i}$ as described above in studies with microelectrodes (Helman and Fisher, 1977). Accordingly, we have assumed this identity to be valid in the present studies, and the calculations of the resistances reflect this.

RESULTS

Studies of $I_{\tau}-V_{\tau}$ Relationships

Previous work from our laboratory (Davies, 1973; Fisher and Helman¹) which involved studies of the transepithelial current-voltage relationships of frog skin showed that despite large decreases of the $I_{se}$ with $10^{-4}$ M ouabain, the changes of $E_{Na}$ as estimated from the breaks at voltages $E_{J}$ (see Fig. 1) were relatively small, in the vicinity of 20%. To explain the entire decrease of the $I_{se}$ it was necessary to suggest that the electrical resistance to transepithelial Na transport was increased by ouabain. This suggestion was in accord with previous measurements of the effect of ouabain on the Na influx at the apical barrier of the skin (Biber, 1971; Erlij and Smith, 1973) and on the osmotic behavior of the frog skin

¹ Fisher, R. S., and S. I. Helman. Unreported results.
(MacRobbie and Ussing, 1961). Since the effects of ouabain on $E_1$ were somewhat unexpected, we report in detail one set of experiments.

In these studies, skins were incubated in a sulfate-Ringer solution containing $5 \times 10^{-7}$ M amiloride in the outer solution. This causes, as reported previously, a partial inhibition of the $I_{sc}$ and a complete linearization (see Fig. 1) of the $I_\tau-V_\tau$ plots between voltages $-40$ mV and the $E_1$ (Macchia and Helman, 1974; Helman and Fisher, 1977; Macchia, 1977). Under these circumstances, the transepithelial electrical model of Fig. 2 describes the behavior of the skin. It follows directly from the slope resistances $R_1$ and $R_\alpha$ ($\Delta V_\tau/\Delta I_\tau$) that

$$R_\alpha = [(R_\alpha R_\alpha)/(R_\alpha - R_\beta)] = E_1/I_{sc},$$

and

$$R_\alpha = (R_\alpha R_\alpha)/(R_\alpha - R_\beta),$$

where $R_\beta$ is the shunt resistance. It also follows directly that if $E_1 = E_{Na}$, the transepithelial current is via $R_\beta$ alone when $V_\tau > E_1$. Thus, $R_\beta = E_1/I_\tau$ (Macchia and Helman, 1974; O’Neil and Helman, 1976; Helman and Fisher, 1977; Macchia, 1977).

**Ouabain on $E_1$ and $I_{sc}$**

A typical study consisted of the determination of six to nine control $I_\tau-V_\tau$ relationships at intervals of 10 min. A summary of mean control parameters is given in Table I for skins exposed to $5 \times 10^{-7}$ M amiloride. After $10^{-4}$ M ouabain was added to the inner solution, $I_\tau-V_\tau$ relationships were determined at intervals of 5 min for up to 3 h. Within 20–40 min, $E_1$ fell ~20% from control and remained essentially constant thereafter (Fig. 3). The $I_{sc}$ fell initially to ~60% of control within 60 min followed thereafter by a secondary decrease toward zero.

It should be noted for these particular studies with skins exposed to $5 \times 10^{-7}$ M amiloride in sulfate Ringer that the time required for ouabain to act on the $I_{sc}$ was relatively long (see later). The reason(s) for this are unknown but the
prolonged duration of action taken together with an especially high shunt resistance averaging 39,000 Ω·cm² permitted long-term observation of $I_T - V_T$ relationships with excellent resolution of the breaks at $E_1$.

**Ouabain on Transepithelial Resistances**

No significant changes of the slope resistances $R_1$ and $R_2$ were observed for the first 40 min of ouabain action (Fig. 4). Between 40 and 60 min, both $R_1$ and $R_2$ decreased markedly to new stable values. As noted above, the shunt resistance was especially high, and as shown in Fig. 5, the shunt conductance, $G_s$, tended to increase but not significantly during the first 40 min. Thereafter, the $G_s$ increased markedly (∼14 times control value) during the ensuing 40 min. Consequently, the decreases of $R_1$ and $R_2$ shown in Fig. 4 can to a large part be attributed to a large decrease of the shunt resistance, $R_s$. Such an observation is compatible with the finding of Biber and Mullen (1977) and this laboratory that ouabain causes large increases of the unidirectional fluxes (inside to outside) of Na and sucrose (Davies, 1973).

Calculation of the values of $R_A^i$ and $R_A^o$ indicated as shown in Fig. 6 that ouabain caused large but selective increases of these resistances. During the first 40–60 min, the resistances increased slowly, but thereafter the $R_A^o$ was increased considerably more than the $R_A^i$.

In summarizing the results of these studies, the data can be interpreted to
indicate that ouabain causes first a small decrease of the $E_{Na}$ as estimated from values of $E_1$, followed by secondary increases of the $R_k$ and $G_s$, occurring predominantly at a time when the $E_1$ appears rather constant.

**Studies with Microelectrodes**

In order to permit an assessment of the changes occurring individually at apical and basolateral membranes, intracellular microelectrodes were used to monitor the intracellular voltage. This also permitted us to obtain estimates of the specific resistances of these barriers to ion transport. Shown in Table II are the control parameters (before ouabain) of 34 skins subjected to study with microelectrodes. Of these, 12 skins were exposed continuously to $10^{-4}$ M amiloride and were considered to be non-Na transporting. The mean $I_{sc}$ of the 22 Na-transporting skins was 44.2 $\mu$A/cm². The $V_{sc}$ averaged $-103.6$ mV and the $E_1$

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Changes of slope resistances $R_1$ and $R_2$ of the $I_{sc}-V_{sc}$ relationships. Values are mean ± SE as percentage of control.

averaged $121.2$ mV. The $\%R_o$ averaged $85.7\%$ and so the outer barrier accounted for a very large fraction of the transcellular resistance. For the skins exposed to $10^{-4}$ M amiloride, the $I_{sc}$ averaged 1.1 $\mu$A/cm², the $V_{sc}$ averaged $-111.2$ mV, and the $E_1'$ averaged $114.7$ mV. With amiloride present in the outer solution, essentially all of the transcellular resistance could be attributed to the outer barrier since the $\%R_o$ averaged $97\%$. For Na-transporting skins, the $R_k'$ was calculated to be $2.470 \Omega$cm², and as expected, amiloride caused the mean value of $R_k'$ to increase to $104,000 \Omega$cm².

**Ouabain on Na-Transporting Skins: Effects on $V_{sc}$, $E_1'$, $I_{sc}$, $R_k'$, and $R_k$**

In all studies, the responses to ouabain could be characterized by two phases to be referred to as rapid and slow phases. As shown in Fig. 7 (see also Figs. 8 and 9), after a delay of a few minutes (ouabain to reach inner chamber and diffuse through corium to the inner barrier), the voltages $V_{sc}$ and $E_1'$ fell rapidly within 5–10 min to mean values of 61.9 and 64.0% of their pre-ouabain control values (Table III) for skins bathed with 2.4 [K]c. Thereafter, the voltages most often
continued to decline slowly, although occasionally the $E'_1$, as shown in Fig. 7, tended to increase during the slow phase. The $I_{sc}$ fell abruptly during the rapid phase to $\sim$60.6% of control and continued to decline during the slow phase of response. At 60 min the $I_{sc}$ had fallen to a mean of 18.3% whereas the $V'_{sc}$ and $E'_1$ remained elevated at 51.6 and 63.1% of their respective pre-ouabain control values (Table III).

According to the interpretation of the above studies of the $I_{sc}$-$V'_r$ relationships, it was anticipated that, in part, inhibition of the $I_{sc}$ with ouabain was mediated via changes of the $R'_o$. To examine this premise with regard to outer and inner barriers, the $R'_o$ and $R'_i$ were calculated with Eqs. 1 and 2. The results of a typical experiment are shown in Fig. 7 (see also Figs. 8 and 9 at reduced $[K]_i$) and are summarized in Table III. As soon as $I_{sc}$ began to decrease (rapid phase), the $R'_i$ increased to values considerably above control with little or no change of the resistance of the outer barrier, $R'_o$. During the slow phase, both the $R'_i$ and $R'_o$ increased markedly to values approximately 14 and 10 times control, respectively, at 60 min for skins bathed with 2.4 $[K]_i$. Indeed, the rapid phases of the response to ouabain were associated with decreases of the voltages $V'_{sc}$ and $E'_1$ and selective increases of $R'_o$. The slow phases were characterized predominantly by changes of the resistances of both apical and basolateral barriers. It should be recalled that since $R'_o >> R'_i$, the decrease of the $I_{sc}$ is more likely attributed to the increase of $R'_o$ of the outer barrier than to the increase of $R'_i$.

**Ouabain in the Presence of Reduced $[K]_i$**

Additional studies were done with ouabain after the $[K]$ of the inner solution was reduced to either 0.5 or 0.0 mM. As shown in Figs. 8 and 9, reduction of
Figure 6. Changes of $R'_A$ and $R''_A$ with $10^{-4}$ M ouabain. Values are mean ± SE as percentage of control.

Table II

CONTROL PARAMETERS FOR SKINS BATHED WITH 2.4 [K]$_i$, CI-HCO$_3$

| Parameter       | Control           | 10$^{-4}$ M Amiloride |
|-----------------|-------------------|-----------------------|
| $I_o$ (mA/cm$^2$) | 44.2 ± 4.9        | 1.1 ± 0.45            |
| $V_o$ (mV)      | -105.6 ± 2.6      | -111.2 ± 2.7          |
| $E'_i$ (mV)     | 121.2 ± 2.4       | 114.7 ± 2.4           |
| $%R_A$          | 85.7 ± 1.9        | 97.0 ± 1.0            |

[K]$_i$ caused the voltages $V_o$ and $E'_i$ to increase. Summarized in Table IV are the pre-ouabain control values of skins bathed with 0.5 and 0.0 [K]. As for skins bathed with 2.4 [K]$_i$, ouabain caused the voltages to fall at first rapidly followed by a slow phase of voltage decline. In these studies the rate of voltage decrease during the rapid phase was considerably faster, especially in those skins bathed with 0 [K]$_i$ (see below). Correspondingly, the $R_A$ was increased selectively during

* No attempt was made to regulate precisely the flow rate through the inner chamber, and consequently, the time of appearance of the ouabain in the inner chamber and its concentration buildup to $10^{-4}$ M was variable. In addition, because of the appreciable unstirred layer consisting primarily of the corium of the skin (~500 μm) the appearance of ouabain at the functional inner barrier would be expected to require in the order of 1-2 min depending, of course, on the thickness.
FIGURE 7. Effect of ouabain on electrical parameters of a Na-transporting skin. 
$[K]_i = 2.4$ mM. Note rapid and slow phases of the responses. $10^{-4}$ M ouabain was 
added to the inner solution reservoir at time zero. The delay in response is in part 
attributed to the time required for the ouabain to reach the inner chamber, to mix 
within the chamber, and to diffuse through the corium to the functional inner 
barrier. Note also the immediate increase of $R_i$ with little or no change of $R_{o'}$. 

The rapid phase with little or no change of the $R_{o'}$, and thereafter both 
resistances increased markedly during the slow phases of the response to 
ouabain (Table III).

In order to quantitate the rapidity of the voltage responses to ouabain, we 

of the corium in any particular skin and the effective diffusion constant of ouabain in this layer. 
Consequently, it was not surprising to observe delays usually in the range of 2–6 min before changes 
in electrical parameter values were observed. Because of the existence of unstirred layers, it remains 
unknown whether the rate of decline of the voltages reflects the rate at which the ouabain 
concentration increases at the functional inner barrier or the rate at which the pump sites bind 
ouabain. Assuming all of these factors being equal, it was observed that the rate of fall of the 
voltages was more rapid when the $[K]_i$ was reduced from 2.4 to 0.5 and 0 mM. Such an observation 
would be compatible with facts established elsewhere that the rate of inhibition of the (Na + K)- 
ATPase by ouabain varies with the $[K]$ of the incubation media (see review by Glynn and Karlish, 
1975).
### Table III

**EFFECT OF OUABAIN ON Na-TRANSPORTING SKINS (PERCENTAGE OF CONTROL)**

|       | $I_w$  | $V_L$  | $R'_C$ | $R'_I$ | $R_s$  | $R'_A$ |
|-------|--------|--------|--------|--------|--------|--------|
|       |        |        |        |        |        |        |
| 2.4 [K], (n = 6) |        |        |        |        |        |        |
| Rapid phase | 60.6±4.9 | 61.9±1.3 | 64.0±1.6 | 107±12 | 179±34 | 119±12 |
| Slow phase, min |        |        |        |        |        |        |
| 15     | 45.6±3.9 | 60.8±1.0 | 64.3±1.6 | 161±18 | 210±36 | 166±16 |
| 30     | 28.6±5.7 | 57.8±3.1 | 62.6±4.7 | 291±55 | 368±76 | 308±60 |
| 45     | 21.8±7.8 | 55.9±4.0 | 63.5±5.6 | 568±108 | 718±241 | 681±207 |
| 60     | 10.3±9.5 | 51.6±4.1 | 68.1±7.3 | 1,059±459 | 1,575±648 | 1,143±555 |
| 0.5 [K], (n = 7) |        |        |        |        |        |        |
| Rapid phase | 55.5±5.2 | 67.2±5.9 | 70.1±4.2 | 120±10 | 277±48 | 151±10 |
| Slow phase, min |        |        |        |        |        |        |
| 15     | 59.2±4.9 | 65.5±6.0 | 67.6±4.4 | 160±15 | 301±49 | 170±15 |
| 30     | 28.6±5.8 | 65.1±6.6 | 64.7±4.8 | 258±40 | 435±83 | 289±40 |
| 0 [K], (n = 5) |        |        |        |        |        |        |
| Rapid phase | 36.4±4.4 | 46.2±5.6 | 52.6±5.5 | 104±20 | 304±26 | 120±25 |
| Slow phase, min |        |        |        |        |        |        |
| 15     | 50.5±9.1 | 47.4±3.9 | 52.2±4.9 | 156±13 | 567±45 | 172±16 |
| 30     | 17.5±2.3 | 47.1±4.7 | 48.5±7.2 | 187±16 | 1,039±590 | 236±92 |
| 45     | 11.0±1.9 | 42.2±6.6 | 46.4±8.5 | 422±114 | 800±297 | 468±144 |
| 60     | 8.3±2.5 | 40.3±3.1 | 47.4±8.3 | 513±140 | 1,470±752 | 682±218 |

For purposes of summary, values were taken at the end of the rapid phase and at 15, 30, 45, and 60 min after addition of 10⁻⁴ M ouabain to the inner solution reservoir. Means ± SE.

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**Figure 8.** Effect of ouabain on electrical parameters in the presence of 0.5 [K]. Note rapid and slow phases and large selective increases of $R_I$ during rapid phase.
FIGURE 9. Effect of ouabain on electrical parameters in the presence of 0 [K],. In this particular experiment $I_{m}$ was not constant during control and experimental periods. Nevertheless, ouabain caused changes of voltage and resistance as observed before. Note rapid and slow phases and selective increases of $R_{t}$ during rapid phase.

TABLE IV
EFFECT OF REDUCED [K], ON TRANSPORT PARAMETERS

| [K], | $I_{m}$ | $V_{T}$ | $E_{r}$ | $R_{t}$ | $R_{1}$ |
|------|--------|--------|--------|--------|--------|
| 2.4 [K], | 57.9±14.4 | -109.9±2.5 | 121.1±3.5 | 2.92±0.49 | 0.18±0.33 |
| 0 [K], | 41.3±16.0 | -130.2±1.9 | 145.9±2.7 | 4.77±1.01 | 0.45±0.17 |
| 0/2.4 | 0.69±0.16 | 1.15±0.02 | 1.19±0.03 | 2.42±0.41 | 2.22±0.48 |

chose to determine the time required, $\tau^{*}$, for the voltages to fall from 10-90% of the entire change of voltage observed during the rapid phase (100%). Despite the uncertainty of the meaning of $\tau^{*}$, it was clear that reduction of [K], resulted in more rapid changes of the voltage in response to ouabain (see Table V). Moreover, these studies with reduced [K], made it especially clear that within a
period of time of, at most, a few minutes ouabain exerted a profound effect at
the inner barrier leading to changes of intracellular voltage and large selective
increases of the $R_{\infty}$.

**Ouabain-Inhibitable Conductance of Inner Barrier**

If we assumed that the action of ouabain on voltage and resistance is confined in
the first few minutes to the inner barrier, it was possible to calculate from the
changes of $R_{\infty}$ (rapid phase) the "ouabain-inhibitable conductance." For skins

**TABLE V**

VALUES OF $\tau^*$

| Amiloride-treated skin | Na-transporting skins |
|------------------------|-----------------------|
| $\Delta E'$ | $\Delta E''$ |
| 2.4 [K] | 8.7±0.9 (6) | 4.5±0.4 | 4.7±0.5 (6) |
| 0.5 [K] | 5.2±0.9 (6) | 5.8±0.4 | 4.1±0.5 (7) |
| 0 [K] | 5.2±0.9 (6) | 1.8±0.1 | 2.6±0.5 (5) |

Means ± SE (n). Values in minutes.

**TABLE VI**

EFFECT OF OUABAIN ON $R_{\infty}$ (RAPID PHASE)

| C | E | E/C | C | E | E/C | C | E | E/C |
|---|---|-----|---|---|-----|---|---|-----|
| 2.4 [K] | 0.5 [K] | 0 [K] |
| Ohm$^*$ | Ohm$^*$ | Ohm$^*$ | Ohm$^*$ | Ohm$^*$ | Ohm$^*$ |
| 150 | 100 | 0.67 | 1,200 | 1,200 | 1.00 | 490 | 1,100 | 2.24 |
| 670 | 700 | 1.04 | 650 | 1,200 | 1.90 | 1,100 | 5,100 | 2.82 |
| 550 | 950 | 1.75 | 370 | 730 | 1.97 | 290 | 850 | 2.98 |
| 600 | 1,300 | 2.17 | 180 | 530 | 2.94 | 185 | 650 | 3.51 |
| 250 | 550 | 2.20 | 300 | 940 | 3.13 | 190 | 700 | 3.68 |
| 620 | 1,800 | 2.90 | 410 | 1,450 | 3.54 | 150 | 730 | 4.87 |

Mean 473 925 1.79 463 969 2.76 451 1,280 3.04
± SE ±89 ±240 ±0.34 ±137 ±124 ±0.48 ±171 ±462 ±0.26

Abbreviations: C, pre-ouabain control; E, after-ouabain (rapid phase) experimental.

bathed with 2.4, 0.5, and 0.0 [K]$_i$ solutions, the ouabain-inhibitable conductance
averaged 28.4, 54.6, and 66.1% of the conductance (pre-ouabain = 100%) of the
inner barrier, respectively (Table VI). Thus, a rather large fraction of the
conductance of the inner barrier was ouabain sensitive.

**Ouabain on Amiloride-Treated Skins**

To assess directly the effects of ouabain on the voltage at the inner barrier,
studies were done with skins exposed to $10^{-4}$ M amiloride outside. Under these
circumstances, the intracellular voltage can be attributed to events occurring
primarily if not alone at the inner barrier since $R_{\infty}^{\infty} >> R_{\infty}$ and the $I_{se} \rightarrow 0$.

The results of representative studies are shown in Figs. 10 and 11 and are
summarized in Table VII. As in the above studies of Na-transporting skins,
ouabain caused the voltages to fall rapidly at first followed by a slow phase during which the voltages remained at high values for considerable periods of time. Reduction of [K], to 0 mM caused the voltages to increase in magnitude. In six skins the mean values of $V_{oc}^+$ and $E'_t$ were increased from $-112.3 \pm 4.5$ and $113.0 \pm 4.5$ mV to $-135.3 \pm 5.1$ and $137.3 \pm 5.2$ mV, respectively. As can be seen in summary Table VII and Figs. 10 and 11, ouabain caused the voltages to decrease approximately 15-20% during the rapid phase. It was observed consistently, with amiloride-treated skins, that the rate of fall of the voltages (rapid phase) was slower than observed for Na-transporting skins. As shown in

![Graph](image)

**Figure 10.** Effect of $10^{-4}$ M ouabain on $V_{oc}^+$ and $E'_t$ of non-Na-transporting skin ($10^{-4}$ M amiloride outside). Note rapid and slow phases.

Table V, the values of $\tau^*$ averaged 8.7 min for amiloride-treated vs. 4.7 min for Na-transporting skins incubated with 2.4 [K],. Similarly, for skins incubated with 0 [K], the mean values of $\tau^*$ were 5.2 and 2.6 min for amiloride-treated and Na-transporting skins, respectively. Although the reasons for this are not known, ouabain appeared to exert an effect on the voltages at the inner barrier (rapid phase) regardless of the rate of transepithelial Na transport.

**DISCUSSION**

It is well established that the glycoside ouabain acts specifically to inhibit the (Na + K)-ATPase of many cell systems thereby inhibiting active Na transport. In frog skin and other epithelia, it is assumed that, by virtue of the existence of this enzyme in these tissues, the inhibition of active transepithelial Na transport by ouabain can be attributed to the inhibition of the ouabain-sensitive Na pumps, presumably the (Na + K)-ATPase.
It is of interest to note recent reviews by Thomas (1972) and DeWeer (1975) where much discussion has centered on the nature of Na pumps in nonepithelial tissues. The observations cited in these reviews and elsewhere (Ussing et al., 1974; DeWeer and Geduldig, 1978) have led to the idea that the Na pumps could indeed be electrogenic, thereby contributing to the magnitude of the intracellular voltage. Consequently, it would be expected that ouabain inhibition of the pumps would result in a decrease of membrane voltage. In this regard, the results of the present studies are in accord with the idea that the Na pumps of frog skin are electrogenic since, regardless of the state of transport, ouabain

![Graph showing the effect of ouabain on $V_o'$ and $E'_o$ on non-Na-transporting skin in the presence of 0 [K]. The data were collected from three cell impalements.](image-url)

**Figure 11.** Effect of ouabain on $V_o'$ and $E'_o$ on non-Na-transporting skin in the presence of 0 [K]. The data were collected from three cell impalements.

**Table VII**

| Effect of Ouabain on Amiloride-Treated Skins |
|--------------------------------------------|
| $2.4 \text{[K]}_o$ | $0 \text{[K]}_o$ |
| $V_o'$ | $E'_o$ | $V_o'$ | $E'_o$ |

| Time | $V_o'$ (mV) | $E'_o$ (mV) | $V_o'$ (mV) | $E'_o$ (mV) |
|------|-------------|-------------|-------------|-------------|
| Pre-ouabain | $-110.2 \pm 3.0$ | $115.6 \pm 2.4$ | $-155.3 \pm 5.1$ | $137.3 \pm 5.2$ |
| 10-15 min | $-95.1 \pm 5.1$ | $101.7 \pm 3.3$ | $-109.3 \pm 6.7$ | $112.2 \pm 6.9$ |
| 30 min | $-87.5 \pm 4.1$ | $95.2 \pm 3.8$ | $-97.2 \pm 8.8$ | $101.3 \pm 9.2$ |
| 45 min | $-89.0 \pm 2.8$ | $95.1 \pm 4.7$ | $-87.3 \pm 10.7$ | $91.4 \pm 10.8$ |
| 60 min | $-76.2 \pm 12.4$ | $83.3 \pm 11.0$ |

Means $\pm$ SE (n).
caused substantial decreases of intracellular voltage. However, such findings taken together with the changes of membrane resistances raise several fundamental questions with regard to the nature of inner and outer barriers and the electrical coupling that occurs between these transport barriers.

**Resistances of Apical and Basolateral Barriers**

The specific resistances of apical and basolateral barriers were found to change when skins were treated with ouabain. Concurrent with the decreases of $E'_i$ and $V^o_i$ (rapid phase), the $R_i$ was observed to increase markedly with little or no effect on the $R^o_i$. Such findings are consistent with the idea that ouabain inhibits a pump whose conductance contributes appreciably to the $R_i$. It is possible that ouabain exerts a direct effect on the K conductance, $g_K$ (see later), of the inner barrier, but in the absence of evidence to the contrary, it would seem reasonable to believe that in frog skin the increases of $R_i$ with ouabain, at least during the rapid phase, can be attributed to a direct action of ouabain on the pump sites. During the slow phase of the response, the resistances of both apical and basolateral membranes increased markedly and continuously with time. This finding is in line with the observations that, approximately 1 h after ouabain, the permeability to Na entry is markedly reduced (Biber, 1971; Erlij and Smith, 1973). Also, MacRobbie and Ussing (1961) concluded that ouabain caused a pronounced decrease of the passive ion permeability of the inner barrier of frog skin. It follows that, if the pumps are inhibited during the rapid phase, then the secondary increases of $R_i$ and $R^o_i$ during the slow phase can be attributed to changes of the conductances of other ions, most likely K at the inner barrier and Na at the outer barrier.

**Electrochemical Potential of K within the Cell**

According to Koefoed-Johnsen and Ussing (1958), the inner barrier of frog skin was thought to possess a neutral Na-K active exchange process that extruded Na from the cells and pumped K into the cells. Owing to a highly selective passive permeability to K, the K efflux from the cells proceeded down its electrochemical gradient so that at the steady state the rates of active K influx and passive K efflux were the same. Thus, the voltage at the inner barrier, $V_i$, is:

$$V_i = E_K - I_K/g_K,$$

where $E_K$ is the Nernst potential for K, $g_K$ is the passive conductance to K, and $I_K$ is the current carried by K from cell to inner solution. Consequently, the $V_i$ would be expected to be less than or equal to $E_K$ under all transporting conditions if the pumps were neutral or electrogenic. Thus, observation of intracellular voltages less than the $E_K$ are compatible with the existence of an electrogenic Na-for-K exchange pump that exists in parallel with a leak pathway for K.

With regard to the interpretation of the origin of the intracellular voltages of the skin and their relationship to the mechanism of the pumps, it is important to know the magnitude of $E_K$, especially in view of our past and present observations of high intracellular voltages in this tissue. In particular, for Na-transporting skins bathed with 2.4 [K]i, the $V^o_i$ averages near $-100$ mV and in
nontransporting skins (amiloride or low Na outside) the intracellular voltage averages considerably higher (115-130 mV). It is not possible at present to know with absolute certainty the \( E_K \) at the inner barrier. However, if we take the highest values of intracellular K concentration, \([K]_e\), near 130-140 meq/liter, reported in the literature (Aceves and Erlij, 1971; Rick et al., 1978a and b), and assuming an activity coefficient of unity, the \( E_K \) is calculated to be near 102 mV when \([K]_i\) is 2.4 mM. Since the intracellular voltages are essentially constant for considerable periods of time (transporting and nontransporting skins) and presumably the tissues are in a steady state, the \( V^*_i \) would be expected to be less than 102 mV under all conditions. Indeed, for Na transporting skins, the values of \( V^*_i \) are near the \( E_K \) and so taken at face value, given the usual uncertainties, \( K \) seems to be close to equilibrium at the inner barrier. We are, however, compelled to note that in the absence of transepithelial Na transport, where presumably the intracellular Na concentration is lowest, the skins generate their highest intracellular voltages which seem to exceed the highest values of \( E_K \) that can be expected on the basis of the K distribution across the inner membrane. It should be emphasized that acceptance of this idea requires that (a) the tissues exist in a steady state so that the extracellular \([K]\) at the functional inner barrier is the same as the bulk phase, and (b) that estimates of intracellular \([K]\) determined chemically reflect the activity of \( K \) within the cells. To the extent that we have not been able to uncover a systematic error in the measurement of the intracellular voltages, we are compelled to consider model systems of the inner barrier that take into account not only the expected observation that \( V_i \leq E_K \), but also the possibility that \( V_i \geq E_K \) especially when transepithelial Na transport is blocked at its site of entry at the apical barrier. It follows directly that, if \( V_i \geq E_K \) at the steady state, a mechanism must exist at the inner barrier for active extrusion of \( K \) from the cells to the inner solution.

Models of Inner Barrier

Shown in Figs. 12 and 13 are mechanistic and electrical equivalent circuit models, respectively, that are consistent with the observed electrical properties of the Na-transporting cells of frog skin. The new suggestion made here is that, in part, K efflux from cell to inner solution may occur via the pump itself. As indicated in Fig. 12, the pump is electrogenic. Accordingly, the pump generates a net current since cation efflux exceeds cation influx \((r > 1)\). Although it is usually thought that active Na efflux exceeds active K influx, it remains possible that, in part, a portion of the active K influx \((I^P_K)\) is recycled via the pump \((I^P_e)\) to the inner solution thereby decreasing the K flux via the leak pathway \((I^L_K)\). To the extent that the affinity of the pump for Na far exceeds its affinity for K, the cation efflux via the pump would be mainly Na.

Although the intracellular \([Na]\) is not known with certainty (see, for example, Rick et al., 1978a), its concentration is known to fall when Na entry is blocked at the apical barrier of the cells. To the extent that a finite competition might exist between Na and K for pump-mediated efflux (especially when \([K]_c \gg [Na]_c\), it would not be difficult to envision situations where the active K influx, \( I^P_K \), could be recycled exclusively via the pump, \( I^P_e \), so that the \( I^L_K \) was zero or even reversed from its usual direction. In such situations the \( V_i \geq E_K \). Indeed, under
extreme conditions when the \([Na]_c \rightarrow 0\), the \(V_i > E_k\), and this situation would correspond in our studies to skins treated with amiloride or bathed with 0 Na outside. Whether this suggestion is tenable remains to be proven, but for the moment, such a mechanism is capable of encompassing the observations that \(V_i \approx E_k\).

We have chosen the above model, in part because it represents to us the simplest model consistent with present beliefs requiring, at most, the simple additional postulate that the affinity of the pump for Na is not absolute. Indeed, it is possible to advance more complicated theories that, in our view, are not presently warranted. Nevertheless, it will be necessary ultimately to explain the existence of high intracellular voltages that are ouabain-inhibitable under conditions where intracellular Na concentrations are likely to be quite low.

Electrical equivalent circuits of the above models are shown in Fig. 13. The pump is modeled with a Thévenin equivalent consisting of electromotive force (EMF), \(E_{\text{pump}}\), in series with its equivalent conductance, \(g_{\text{pump}}\). Defining \(I_k^{t+}\) positive as indicated by the arrows in Fig. 13, current via the pump, \(I^p\), is:

\[
I^p = I_{Na} - I_k,
\]

and

\[
I_k = I_k^{t+} - I_k^{t-}.
\]

When \(I_k^{t-} \leq I_k^{t+}\), \(I_k\) occurs from cell to inner solution. Thus,

\[
V_i = E_k - I_k/g_k.
\]

If, however, \(I_k^{t-} > I_k^{t+}\), \(V_i > E_k\) since \(I_k\) must occur at the steady state from inner solution to cell interior.
In general, for skins short-circuited, it can be shown that:

$$V_o^p = E_{pump} \left( \frac{g_{pump}}{g_{pump} + g_K + G_{Ra}} \right) + E_K \left( \frac{g_K}{g_{pump} + g_K + G_{Ra}} \right),$$

and so the intracellular voltage will depend not only on the EMFs $E_{pump}$ and $E_K$ but also on the conductances where $G_{Ra}$ is the Thévenin conductance to Na of the apical barrier (see later).

In this regard, it was observed in the present studies that ouabain caused substantial increases of the resistance of the inner barrier, $R_i$. If the $R_i$ can be attributed primarily to the conductances $g_K$ and $g_{pump}$, then it is obvious that the pumps cannot be rheogenic (constant current-like) inasmuch as a rheogenic pump mechanism would require that $g_p < g_K$. Since this appears not to be the case in frog skin, it follows that the current via the pump would be influenced by changes of intracellular voltage however mediated. If we assume that ouabain at $10^{-4}$ M binds specifically and inhibits all pump sites, it follows that the $V_o^p$ and $I_{sc}$ after ouabain are

$$V_o^p = E_K \left( \frac{g_K}{g_K + G_{Ra}} \right),$$

and

$$I_{sc} = E_K(\frac{g_K}{g_K + G_{Ra}}).$$

Clearly, such a situation is transient since the $I_{sc}$ is carried by Na entry at the apical barrier and by K efflux via the basolateral barrier leading ultimately to a dissipation of the Na and K concentration gradients. Although no data are now available on the time-course of change of intracellular Na and K concentrations, it is well established for Na-transporting skins that the Na and K gradients fall within 60-90 min (see, for example, Rick et al., 1978a). It is also noteworthy
that for non-Na-transporting skins (amiloride or 0 [Na] outside) the cells maintain their gradients for Na and K for at least 60-90 min (Rick et al., 1978a). In the context of the models shown here, such an observation would not be surprising by virtue of the electrical requirement that for K to leave the cells at the basolateral barrier, Na entry (current) at the apical barrier must occur in a 1:1 intracellular exchange of Na for K. In this regard, it was observed that for nontransporting skins, the intracellular voltages remained at high values after ouabain consistent with the observations of the persisting Na and K gradients after ouabain reported by Rick et al., 1978a (see also below).

Changes of $V_{so}$ with Ouabain

It would seem straightforward to expect, because of the electrical coupling between outer and inner barrier, that changes of $V_{so}$ would be complicated not only by changes of the resistances but also by changes of the EMFs of outer and inner barriers. Such difficulties are obviated in studies of nontransporting skins (amiloride or 0 [Na] outside), where the changes of $V_{i}$ are due to events occurring primarily if not alone at the inner barrier. Under these circumstances, ouabain caused the $V_{i}$ to fall ~15--20% from control values (2.4 [K] outside) averaging near 95 mV at the end of the rapid phase. From the models shown above, the $V_{i}$ before ouabain is:

$$V_{i} = E_{pump} \left( \frac{g_{pump}}{g_{pump} + g_{K}} \right) + E_{K} \left( \frac{g_{K}}{g_{pump} + g_{K}} \right),$$

and after ouabain assuming $g_{pump} \rightarrow 0$,

$$V_{i} \approx E_{K}. \quad (11)$$

Accordingly, the $V_{i}$ after ouabain might, as a first approximation, estimate the $E_{K}$ which in the present studies was observed to fall in the vicinity of 95--100 mV at the end of the rapid phase. With 2.4 [K] outside the intracellular [K] would be expected to be near 105--127 meq/liter if $E_{K}$ is 95--100 mV. It is unknown, however, to what extent and at what rate intracellular and extracellular concentrations of K change after ouabain and so influence the magnitude of $V_{i}$ under Na-transporting and non-Na-transporting conditions. For the latter case, the changes of intracellular (and extracellular) [K] might be expected to be relatively small since [K] remains elevated for considerable times (at least 60--90 min) after ouabain. However, for Na-transporting skins treated with ouabain, changes of the K gradient could be appreciable due not only to decreases of [K], but also to increases of extracellular [K] at the functional inner barrier of the cells. For example, assuming $I_{sc}$ of 18 $\mu A/cm^{2}$ and equal to the rate of K loss at the inner

---

3 The models suggested here assume that the apical membrane is passively permeable only to Na and that the basolateral membrane is passively permeable only to K. To the extent that large changes of [Cl] on both sides of the tissue and large changes of [Na] in the inner solution cause little or no change of the $V_{so}$, it is assumed at least to a first approximation that the outer barrier is primarily permeable to Na and that the inner barrier is primarily permeable to K as suggested originally by Koefoed-Johnsen and Ussing (1958). Indeed, the inner barrier of frog skin with 10^{-4} M amiloride outside yields changes of $V_{i}$ that average near 65 mV/decade change of [K] outside (Fisher and Helman, 1978).
barrier, it can be calculated that extracellular [K] would increase from 2.4 to 5.2 meq/liter in order for K to diffuse at this rate from the extracellular space to the inner solution through the unstirred layer of the corium of approximately 460 μm in thickness possessing a diffusion coefficient for K of $3 \times 10^{-6}$ cm²/s as estimated in separate studies by Fisher and Helman. Consequently, elevation of extracellular [K] alone from 2.4 to 5.2 meq/liter would cause the $E_K$ to decrease by about 20 mV over and above depolarization of $V_i$ by ouabain inhibition of the pumps. This, in part, may explain why the changes of $V_n'$ after ouabain were considerably greater in Na-transporting vs. non-Na-transporting skins. To test this idea, it will be necessary to study split skins, in the absence of the unstirred layers of the corium.

**Transepithelial Driving Force**

In 1951 Ussing and Zerahn proposed an equivalent circuit wherein the $E_{Na}$ represented the Thévenin EMF for active transepithelial Na transport. It would seem clear that, because it is an equivalent transepithelial parameter, the $E_{Na}$ is not a parameter of the pump mechanism itself. Indeed, as observed here and elsewhere, the voltages at both apical and especially basolateral barriers are sensitive to ouabain and to changes of the [K]ᵢ. If we assume according to our previous observations that the $E_{Na}$ can be estimated equally well from values of $E_1$ ($\eta_T - V_T$ relationship) as well as $E_1'$ (microelectrode determinations), then it would seem clear that the $E_{Na}$ is dependent upon the $E_K$ and $g_K$ of the inner barrier. Indeed, when $I_{sc} \to 0$ (amiloride or 0 [Na] outside), the Thévenin EMF of the inner barrier is equal to $E'$; and so changes of $E_1'$ with ouabain or [K]ᵢ, would be reflected as changes of $E_{Na}$. Perhaps the point to be emphasized is that the $E_{Na}$ defined electrically, cannot be assumed to be the "EMF of the Na pump." In this regard, the $E_{pump}$ in our opinion, better serves this purpose.

As a final note, little is known of the mechanism of Na entry at the apical barrier of the cells. Our own studies have emphasized the existence of electrical rectification at this barrier, and taken together with the known saturable-like phenomenon for Na entry and the additional fact that the $V_n'$ is far from the Na chemical equilibrium potential, it is difficult to know with certainty what model best serves to describe the behavior of this barrier. Our previous studies indicated that the Thévenin equivalent of the apical barrier was a simple resistor ($V_T < E'$), and we have used this equivalent in the present studies ($G_{Ra} = 1/R_R$). To the extent that we have not been able to obtain data in conflict with this idea, the outer barrier has been modeled with conductance $G_{Ra}$, and the calculations reflect this working hypothesis.

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