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Effects of 2-Deoxy-D-Glucose, Amiloride, Vasopressin, and Ouabain on Active Conductance and E_{Na} in the Toad Bladder

C.D.Hong* and A.Essig**

Department of Physiology, Boston University School of Medicine, Boston, Massachusetts

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Summary. The effects of various agents on active sodium transport were studied in the toad bladder in terms of the equivalent circuit comprising an active conductance κ^a, an electromotive force E_{Na}, and a parallel passive conductance κ^p. For agents which affect κ^a, but not E_{Na} or κ^p, the inverse slope of the plot of total conductance κ against short-circuit current I_0 evaluates E_{Na}, and the intercept represents κ^p. Studies employing 5×10^{-7} M amiloride to depress κ^a indicate a changing E_{Na}, invalidating the use of the slope technique with this agent. An alternative suitable technique employs 10^{-5} M amiloride, which reduces I_0 reversibly to near zero without effect on κ^p. Despite curvilinearity of the $\kappa-I_0$ plot under these conditions, κ^p may therefore be estimated fairly precisely from the residual conductance. It then becomes possible to follow the dynamic behavior of κ^a and E_{Na} (in the absence of 10^{-5} M amiloride) by frequent measurements of κ and I_0, utilizing the relationships $\kappa^a=\kappa-\kappa^p$, and $E_{Na}=I_0/(\kappa-\kappa^p)$. 2-deoxy-D-glucose ($7.5\times10^{-3}$ M) depressed κ^a without affecting E_{Na}. Amiloride (5×10^{-7} M) depressed κ^a and enhanced E_{Na}. Vasopressin (100 mU/ml) enhanced κ^a markedly and depressed E_{Na} slightly. Ouabain (10^{-4} M) depressed both κ^a and E_{Na}. All of the above effects were noted promptly; κ^p was unaffected. The "electromotive force of Na transport" E_{Na} appears not to be a pure energetic parameter, but to reflect kinetic factors as well, in accordance with thermodynamic considerations.

Transepithelial sodium transport in the toad bladder is by way of two parallel pathways, active and passive. According to the currently accepted view, the active pathway consists of an apical passive permeability barrier in series with a Na-K activated ATPase (Na "pump") at the basolateral membrane. The rate of active sodium transport across this epithelial tissue can be altered, in principle, by varying any of the following: (1) the permeability of the apical passive barrier; (2) the free energy of the metabolic reaction driving transport; and (3) the function of the pump itself.

* *Present address:* Department of Medicine, University of Cincinnati College of Medicine, Cincinnati, Ohio.

** *Send correspondence to:* Dr. A.Essig, Department of Physiology, Boston University School of Medicine, 80 East Concord Street, Boston, Mass. 02118.

For the analysis of active transepithelial sodium transport many investigators have utilized an equivalent circuit model [5, 10, 13, 24, 25]. In this model the active pathway consists of a conductance element, which we may call κ^a, in series with the electromotive force of sodium transport E_{Na}. The parallel passive pathway may be represented by the conductance κ^p. E_{Na} is commonly considered to be the energetic factor of the active sodium transport system, and investigators have attempted to evaluate it with a variety of techniques [4, 5, 10, 17, 25, 29]. Theoretical arguments, however, suggest that E_{Na} is a composite variable incorporating not only energetic factors, but also kinetic factors [3, 5, 9]. On the other hand, the affinity of nonequilibrium thermodynamics (essentially the negative Gibbs free energy of the metabolic reaction driving active sodium transport) is presumably a pure energetic term [9, 16, 18, 26].

In the present study we observe the effects on the equivalent circuit elements of substances whose mechanism of action is to some extent defined. Where possible, the behavior of E_{Na} is compared with that reported previously for the affinity.

Materials and Methods

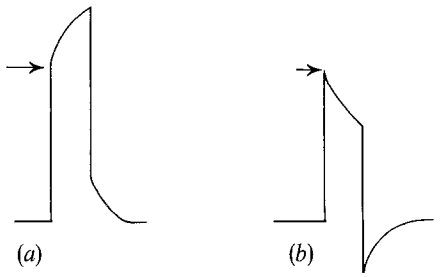
I. Biological Membranes – General Methods

Female toads *(Bufo marinus)* were obtained from the Dominican Republic (National Reagents, Bridgeport, Connecticut). All studies were carried out in paired urinary hemibladders of a single toad. The membranes were mounted in modified Ussing-Zerahn Lucite chambers [25] of 7.54 cm^2 cross-sectional area. Each reservoir was filled with 20 ml of glucose sodium Ringer's solution. The electrical potential difference $\Delta\psi$ was regulated with a voltage clamp, and the current I [mucosa (M) to serosa (S)] was recorded continuously. In order to eliminate tissues with significant edge damage, membranes with open-circuit potentials less than 40 mV 30 min after mounting were discarded. In acceptable membranes the fraction of total conductance attributable to the active pathway κ^a/κ was 0.55 ± 0.03 (mean $\pm$ SEM) ($n = 40$) [17].

II. Parameters of Equivalent Circuit Model

A. Determination of Total Conductance κ

Since the current-voltage relationship is linear over the voltage range of present interest [19], the total conductance κ (mmho cm^{-2}) was evaluated from $-\Delta I/\Delta(\Delta\psi)$, setting $\Delta\psi$ sequentially at $+20$, 0, -20, and 0 mV for 5 sec each. This was done automatically by a timing circuit at 20 or 60 sec intervals, as desired. In about half the instances prior to amiloride, with rapid chart speed the wave form was not square, but showed either an "elbow" (*a*) or a "peak" (*b*), as indicated by the arrows below:



Because of the frequent need for the measurement of multiple values of κ during rapid depression of transport, it was elected to use the elbow (or peak) value of ΔI in the calculation of E_{Na} (*IIDb* below). Control values of E_{Na} evaluated in this manner averaged 117.7 ± 7.2 mV ($n=37$) (mean $\pm$ SEM) as compared with 117.1 ± 8.2 mV ($n=11$) (mean $\pm$ SEM) measured in toads from the same shipment by a steady-state technique employing values of ΔI observed 30 sec after perturbation of $\Delta\psi$ (Lang, Caplan, and Essig, *manuscript in preparation*).

B. Determination of Passive Conductance κ^p

a. Tracer Flux Technique. ^{22}Na fluxes were measured as described previously [17, 19]. In order to measure tracer fluxes accurately in membranes of low passive permeability a 50 mV electrical potential difference was imposed (serosa positive). κ^p (in mmho cm^{-2}) was then taken as

$$\kappa^p = 4.04 \times 10^5 (\overleftarrow{J^*}/\Delta c^*)_{\Delta\psi=50},$$

where $\overleftarrow{J^*}$ represents serosal to mucosal ^{22}Na flux (activity per cm^2/sec) and Δc^* is the concentration difference of ^{22}Na (activity per cm^3) across the membrane.

b. Amiloride Chord Intercept Technique. Values of total conductance κ measured at 1 min intervals were plotted against simultaneously determined values of short-circuit current $I_0 (I_{\Delta\psi=0})$ prior to and following the exposure of the mucosal surface to 10^{-5} M amiloride. Within 5 min following administration of the drug I_0 fell to a steady-state level at 5 ± 2 % of the original level. Since the nature of the steady-state $\kappa - I_0$ relationship was not known in detail over its entire range, κ^p was estimated by drawing a straight line through the two steady-state data points at $t=0$ and $t=5$ min and extrapolating to the intercept at $I_0=0$. The value of κ at the chord intercept was taken as κ^p. Since the $\kappa - I_0$ plot is curvilinear and concave upward (Fig. 1) this extrapolation procedure must lead to underevaluation of κ^p. The resultant errors in the evaluation of κ^a and E_{Na} from Eqs. (2) and (5) (Results) will be discussed below.

κ^p was determined by the above means prior to and following exposure to each test agent.

C. Determination of Active Conductance κ^a

a. *Slope Technique.* κ^a was calculated as the quotient of I_0 and E_{Na} (Eq. (1), Results).

b. *Chord Intercept Technique.* κ^a was calculated as the difference of total and passive conductance (Eq. (2), Results). The value of κ^p, determined by the chord intercept technique at the end of the experiment, was applied to previously determined values of κ. Since control values of κ^a and κ^p were about equal, any error in κ^p would result in a comparable error in κ^a. The error in calculation of κ^a by this means can be estimated by comparing the value of κ^a derived from the chord intercept (which under-estimates κ^p and over-estimates κ^a) with the

value derived from taking as κ^p the value of κ associated with 5 % residual I_0 (which over-estimates κ^p and under-estimates κ^a). In 12 control membranes the mean difference of κ^a determined by these two techniques was 3.9 ± 0.7 % (SEM) of the chord intercept value. This is the largest possible error. In 6 membranes in which an additional very high dosage of amiloride abolished I_0 completely, κ fell further by about 1 to 2 % as I_0 fell from about 5 % of the original level to zero. Thus the over-estimate in κ^a by the chord intercept technique must have been about 2–3 %. In those membranes in which κ^a was enhanced by ADH, the % error was smaller, and in membranes in which κ^a was depressed by amiloride, 2-deoxy-D-glucose, or ouabain, the % error was larger than in the control bladders. In the most extreme case, transient depression of κ^a by amiloride to some 20 % of control level (Fig. 3), the chord intercept technique would over-estimate κ^a by about 10–15 %.

D. Evaluation of the Electromotive Force of Sodium Transport E_{Na}

a. *Slope Technique.* Total conductance κ and short-circuit current I_0 were measured at 20 sec intervals before and during the rapid decrease of I_0 after exposure to 5×10^{-7} M amiloride. The slope of the $\kappa - I_0$ relationship was obtained by regression analysis of the data points, and the inverse of the slope was taken as E_{Na}.

b. *Chord Intercept Technique.* The conductance of the passive pathway κ^p determined at the end of the experiment by the use of 10^{-5} M amiloride was applied to previously determined values of κ and I_0 to calculate E_{Na} from Eq. (5) (Results). Since κ^a is over-estimated by the chord intercept technique, the use of Eq. (5) will under-estimate E_{Na} to the same extent, *i.e.* by some 2–3 % in the case of control tissues. The error would be smaller where κ^a was enhanced by ADH treatment, and larger where κ^a was depressed by test agents (*IIC* above).

III. Experimental Protocol – Chord Intercept Technique

1. After 30 min of incubation at open circuit, the tissue was short-circuited and allowed to stabilize for 2 hr.

2. Following the initial determination of κ, amiloride was added to the mucosal hemichambers of both experimental and control tissues (final concentration, 10^{-5} M). κ was again measured 5 min after the addition of amiloride.

3. The mucosal bathing solution was rapidly but gently changed by simultaneous infusion and suction. Adjustment of the level of the tip of the suction needle minimized transmembrane hydrostatic pressure gradients and tissue damage. About 500 ml of fresh glucose Na Ringer's solution was exchanged within 6 min.

4. The system was allowed to restabilize for 60 min.

5. While measuring κ at 1 min intervals, test agent was added to the appropriate hemichamber. The same volume of solvent was added to the other experimental and control hemichambers.

6. Following an observation period, κ was again determined before and after the use of amiloride, as above.

IV. Materials

The glucose sodium Ringer's solution consisted of glucose (1.0 mM) and Na 115.9, K 2.5, Ca 1.8, Cl 117.8, and HCO_3 2.4 mEquiv/liter (pH 7.6, 233 mOsm/kg H_2O). Solutions were prepared daily from concentrated glucose stock solution and stock Na Ringer's solution. ^{22}Na was obtained from New England Nuclear, Boston, Mass. Amiloride was a gift from Dr.

C. A. Stone of Merck, Sharp and Dohme Company. Also employed were antidiuretic hormone (Vasopressin: Pitressin, Parke-Davis), 2-deoxy-D-glucose (Sigma Chemical Company), and ouabain (Calbiochem.).

V. Analysis of Data

Data were expressed as the arithmetic mean ± standard error of the mean (SEM). For the analysis of temporal changes the variables were converted into ratios $r(x)=x_t/x_{t=0}$, where t refers to time in minutes, and the geometrical mean and standard error of the mean of this ratio were calculated. Results in paired hemibladders were compared by Student's t test [23]. The effect of test agents on κ^p was analyzed by the Mann-Whitney U test [22]. (For geometric mean ratios the quantity [Antilog $(\overline{\log r}+\text{SEM})$ − Antilog $(\overline{\log r}-\text{SEM})$]/2 was taken as the SEM.)

Results

Evaluation of E_{Na}: Slope technique (5×10^{-7} M amiloride)

In the equivalent circuit model for active sodium transport, Na^+ ions are considered to be actively transported through channels of conductance κ^a by a Na^+ pump of electromotive force E_{Na}. Na^+ and other ions traverse the tissue passively through a parallel pathway whose conductance we designate κ^p [24, 25, 29]. Thus, paraphrasing Yonath and Civan, the short-circuit current $I_{\Delta\psi=0}$ is given by

$$I_0=\kappa^a E_{Na}, \tag{1}$$

and the total conductance is given by

$$\kappa=\kappa^a+\kappa^p. \tag{2}$$

Combining Equations (1) and (2) gives Yonath and Civan's relationship between the total conductance and the short-circuit current:

$$\kappa=\kappa^p+(1/E_{Na})I_0. \tag{3}$$

In their approach Yonath and Civan plotted the dependence of κ on I_0 following the administration of antidiuretic hormone (ADH). Assuming that ADH stimulates sodium transport only as a consequence of enhancement of κ^a, and is without effect on κ^p or E_{Na}, it is seen that the plot will be linear and the inverse of the slope can be taken as E_{Na}.

In our initial attempts to study the behavior of E_{Na} we adopted a similar technique. However, rather than using ADH to alter transport we used 5×10^{-7} M amiloride, for the following reasons. It is known that amiloride acts only on the outer permeability barrier, reducing the permeability of

Table 1. Effects of 2-deoxy-D-glucose, amiloride, and antidiuretic hormone on I_0, κ^a, and E_{Na}, as estimated by the slope technique[a]

Agent	t (min)	n	I_{0t}/I_{00}	κ^a_t/κ^a_0	$E_{Na\,t}/E_{Na\,0}$
2-DG (7.5×10^{-3} M)	120	6	0.20 ± 0.02[1]	0.21 ± 0.04[1]	0.94 ± 0.09
Glucose (7.5×10^{-3} M)	120	6	0.74 ± 0.06	0.78 ± 0.08	0.95 ± 0.05
Amiloride (5×10^{-7} M)	150	7	0.73 ± 0.11	0.43 ± 0.07[2]	1.78 ± 0.13[1]
Control	150	7	0.91 ± 0.09	0.89 ± 0.09	1.02 ± 0.03
ADH (100 mU/ml)	30	6	1.05 ± 0.09	1.48 ± 0.14[3]	0.71 ± 0.07[4]
Control	30	6	0.93 ± 0.03	1.04 ± 0.04	0.90 ± 0.03

[a] The period of exposure to the agents prior to time t was 60 min for 2-DG and glucose, 150 min for amiloride, and 30 min for ADH. The data are expressed as the geometric mean $\pm$ SEM of $r(x)=x_t/x_{t=0}$, where t is time in minutes. Glucose treated hemibladders served as controls for the 2-DG experiment. The behavior of this group was not significantly different from that of the control group of the amiloride experiment. Significant differences between control and experimental tissues:

[1] $p<0.001$. [2] $p<0.005$. [3] $p<0.02$. [4] $p<0.05$.

this membrane to Na^+, without apparent direct effect on the pump [1, 2, 6, 7, 20, 21]. Amiloride fails to affect the passive serosal to mucosal radioactive Na fluxes, indicating that there is no effect on κ^p. (The ratio of $S\rightarrow M$ tracer Na fluxes after and before 90 min of exposure to 10^{-6} M amiloride was 1.04 ± 0.03, as compared to the corresponding value of 1.04 ± 0.06 in paired control hemibladders ($n=6$).) The action is rapid, with maximum inhibition of I_0 within 5 min. At dosages less than 10^{-6} M, I_0 is inhibited by less than 75 %, and the effect is reversible. For these various reasons it was felt that it should be feasible to use amiloride to evaluate E_{Na} in essentially the same way as with ADH, and to compare the results obtained by these different means.

With this technique we studied first the effects of 2-deoxy-D-glucose (2-DG), an inhibitor of carbohydrate metabolism and ATP production [27, 28]. This agent has been shown to depress active sodium transport and the thermodynamic affinity in the frog skin [15, 16]. As is seen in Table 1, 7.5 mM 2-deoxy-D-glucose caused a marked inhibition of I_0, with a parallel reduction in κ^a. Of special interest was the lack of apparent effect on E_{Na}.

We next examined the effects of prolonged exposure to amiloride. For this purpose, following the use of 5×10^{-7} M amiloride to determine the initial values of E_{Na} and κ^a amiloride was removed only from the control chambers, whereas the experimental membranes continued to be exposed to the drug for 150 min. At this time the control tissue was treated with

1.0×10^{-6} M amiloride and the concentration of amiloride in the experimental chamber was increased to 1.0×10^{-6} M. This caused sufficient depression of κ and I_0 in each case to permit the evaluation of the final values of κ^a and E_{Na} (Table 1). Although 5×10^{-7} M amiloride resulted initially in a substantial depression of I_0, with the passage of time there was sufficient recovery that at 150 min I_0 differed insignificantly in the experimental and control membranes. Nevertheless, E_{Na} was substantially greater and κ^a substantially less in the experimental membranes than in control membranes.

A similar approach was applied to study of the effects of ADH (100 mU/ml). Since the second measurements were made 30 min after the administration of ADH, the stimulation of I_0 by ADH had subsided in most cases, and the mean I_0 was not significantly greater in the experimental than in the control group. Nevertheless, κ^a appeared significantly greater and E_{Na} significantly less than in the control group.

During the course of this study our attention was drawn to the confusing behavior of the intercept of the projection of the $\kappa - I_0$ plot of Eq. (3), which if E_{Na} is constant should give κ^p. Following ADH treatment, the intercepts obtained by the use of low concentrations of amiloride, which inhibited I_0 to some 50–60 % of the original level, were smaller than the baseline intercepts. Furthermore, on occasion the intercept became negative. (In an attempt to avoid falsely low estimates of E_{Na}, studies with negative intercepts were excluded from Table 1.)

Interpretation of the $\kappa - I_0$ relationship

Because a negative κ^p is physically meaningless, at this point we re-examined the nature of the $\kappa - I_0$ relationship. By differentiation of Eq. (3) we obtain the slope $d\kappa/dI_0$. Assuming that κ^p remains constant (*i.e.* $d\kappa^p/dI_0 = 0$) during the alteration of I_0 with a given agent,

$$\frac{d\kappa}{dI_0} = \frac{1}{E_{Na}} - \frac{I_0}{E_{Na}^2}\frac{dE_{Na}}{dI_0} = \frac{1}{E_{Na}}\left(1 - \kappa^a \frac{dE_{Na}}{dI_0}\right). \tag{4}$$

It is seen that E_{Na} is given by the inverse slope of the $\kappa - I_0$ plot only if E_{Na} is unaffected by the experimental means employed to change I_0. In general the relationship may be more complex and the value of E_{Na} estimated from $d\kappa/dI_0$ will then be either too small or too large, according as dE_{Na}/dI_0 is negative or positive respectively. For the same reason, the intercept of the projection of a linear portion of the $\kappa - I_0$ plot would be either less than or greater than κ^p, respectively.

EFFECT OF ANTIDIURETIC HORMONE AND AMILORIDE ON THE RELATIONSHIP OF CONDUCTANCE TO SHORT CIRCUIT CURRENT IN THE TOAD BLADDER

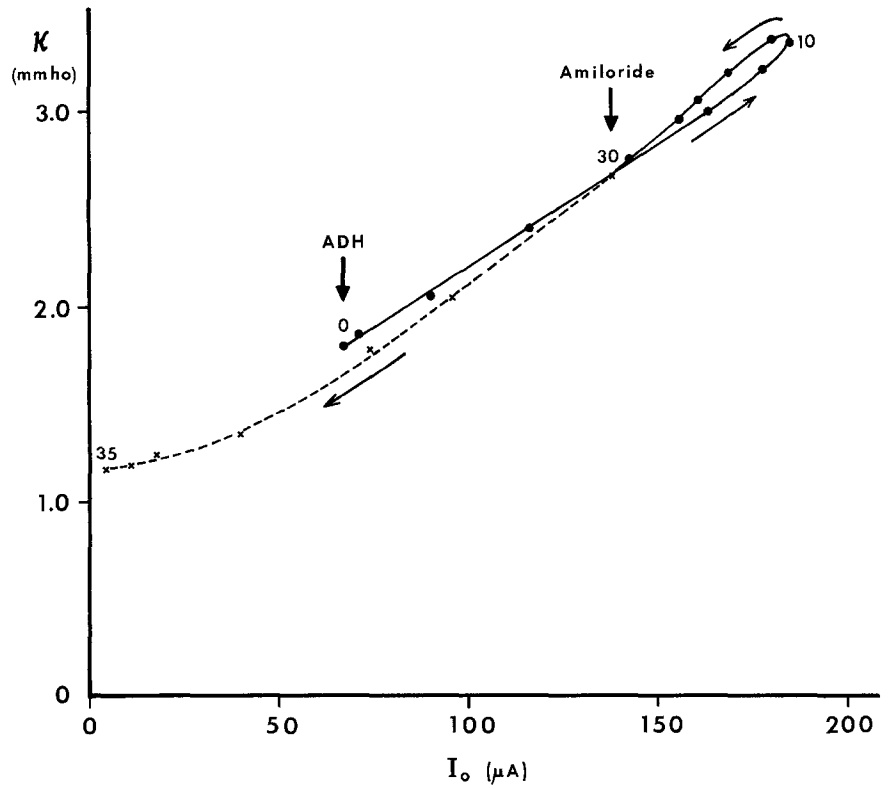


Fig. 1. Relationship of conductance κ to short-circuit current I_0 in a toad bladder of 7.54 cm^2 area treated with ADH (100 mU/ml) at time zero (●——●) and then with amiloride (10^{-5} M) at 30 min (× ------ ×). Final observation at 35 min

The significance of these considerations may be appreciated by examining Fig. 1, which demonstrates the $\kappa - I_0$ relationship of a toad bladder in which I_0 was first stimulated by ADH, then depressed by amiloride. In this case a high dosage of amiloride was employed in order to examine the $\kappa - I_0$ relationship over a wide range. Several features are noteworthy. It is seen that the plot appeared to be impressively linear during the early response to both ADH and amiloride. Subsequently, however, with

approach to maximum response, in each case marked departure from linearity became evident; with reversal of the ADH effect this resulted in the formation of a loop. Since control studies demonstrated that neither test agent affected κ^p, the demonstration of curvature of the $\kappa - I_0$ plot is consistent with the interpretation above that both agents affect E_{Na}, with stimulation of transport by ADH depressing E_{Na} and inhibition of transport by amiloride enhancing E_{Na}.

Although the demonstration of curvature of the $\kappa - I_0$ plot in the face of constancy of κ^p is unequivocal evidence for an effect of a test agent on E_{Na}, the converse inference should not be drawn. Consideration of Eq. (4) shows that in order for a region of the $\kappa - I_0$ plot to be linear (*i.e.* for $d\kappa/dI_0$ to be constant) it is not necessary that E_{Na} be constant (*i.e.* $dE_{Na}/dI_0 = 0$), but only that the quantity $(1/E_{Na})(1 - \kappa^a dE_{Na}/dI_0)$ be constant. Thus the observation that the $\kappa - I_0$ plot appears to be linear following the administration of an agent in no way guarantees that the agent has no effect on E_{Na}. Indeed, analysis of Fig. 1 indicates that in this experiment E_{Na} decreased promptly following the administration of ADH, well before the $\kappa - I_0$ plot became noticeably curvilinear. This can be deduced by noting that the intercept of the projection of the early ADH plot is appreciably lower than that of the amiloride curve. Since I_0 in the presence of amiloride at 35 min was near zero, the intercept of the amiloride curve must give a fairly accurate value of κ^p. Hence the finding that the intercept of the projected ADH plot is lower must reflect steepness of the slope attributable to a negative value of dE_{Na}/dI_0 in the presence of ADH.

Evaluation of E_{Na}: *Intercept technique* (10^{-5} M *amiloride*)

The above findings make for uncertainty in the determination of E_{Na} by the amiloride-slope technique, since even when the $\kappa - I_0$ relationship appears to be linear, independent evidence is needed to assure that the inverse slope evaluates E_{Na}. We therefore sought other convenient means for this purpose. One standard means involves the concurrent measurement of κ^p, using radioactive tracer techniques, and total conductance κ. This permits the calculation of κ^a from Eq. (2); knowledge of I_0 then gives E_{Na} from Eq. (1) [11, 17, 19, 24]. Thus,

$$E_{Na} = I_0/(\kappa - \kappa^p). \tag{5}$$

The rapid and dramatic effects of amiloride suggest that the use of this agent might provide a more convenient means of evaluating the passive

conductance than a radioactive tracer technique. Since amiloride depresses I_0 as a consequence of depressing Na^+ entry into the active pathway, the conductance remaining after the abolition of I_0 by amiloride should represent κ^p. Reversibility of the effect would permit intermittent exposure to amiloride with repeated determination of κ^p during the course of an experiment.

In practice it proved impractical to abolish I_0 completely with amiloride, since the effect was not then fully reversible. Furthermore, the extensive washing necessary to remove the high concentration of amiloride employed traumatized the membranes. It was found, however, that the use of 10^{-5} M amiloride is practical. This concentration reduced I_0 to a mean of 5 ± 2 % of the original level, permitting an adequately accurate value of κ^p to be obtained by extrapolation of the chord of the $\kappa - I_0$ plot. The effect was rapid, reaching its maximum within 5 min, and was reversible on removal of the drug, without undue tissue damage. (Mean $\Delta\kappa^p$ following the washing procedure, evaluated by radioactive tracer techniques, was 0.021 ± 0.006 mmho/cm^2 ($n = 31$), some 10 % of the mean initial κ^p value. The value of $\Delta\kappa^p$ was unrelated to the original level of κ^p.)

Before applying this method for the evaluation of E_{Na} it is of course necessary to assess the possible effect of high concentrations of amiloride on the passive pathway. This was done by measurements of serosal to mucosal radioactive sodium tracer fluxes. It was found that, as previously with 10^{-6} M amiloride, 10^{-5} M amiloride fails to affect κ^p significantly. (The ratio of $S \rightarrow M$ tracer Na fluxes after and before 90 min of exposure to 10^{-5} M amiloride was 1.21 ± 0.10, as compared to the corresponding value of 1.09 ± 0.10 in paired control hemibladders ($n = 8$).)

Further analysis tested how accurately the amiloride technique evaluates the passive conductance. This was done by comparing values of κ^p measured by the use of the 10^{-5} M amiloride chord intercept technique with those measured concurrently in the same tissues by means of $S \rightarrow M$ ^{22}Na tracer flux [19]. In 14 studies the mean values of κ^p obtained from the two techniques were 0.213 and 0.202 mmho/cm^2 respectively. The difference (0.011 ± 0.012 mmho/cm^2) was insignificant ($p > 0.30$). [In contrast, values of κ^p measured by the use of the 10^{-5} M amiloride chord intercept technique differed from values measured 12–35 min earlier in the same hemibladders by extrapolation of the slope of the $\kappa - I_0$ plot following exposure to 100 mU/ml ADH. In 10 studies the mean values were 0.175 mmho/cm^2 (chord technique) and 0.157 mmho/cm^2 (slope technique). The difference (0.018 ± 0.006 mmho/cm^2) was significant ($p < 0.02$).]

With the availability of a convenient means to evaluate κ^p, frequent

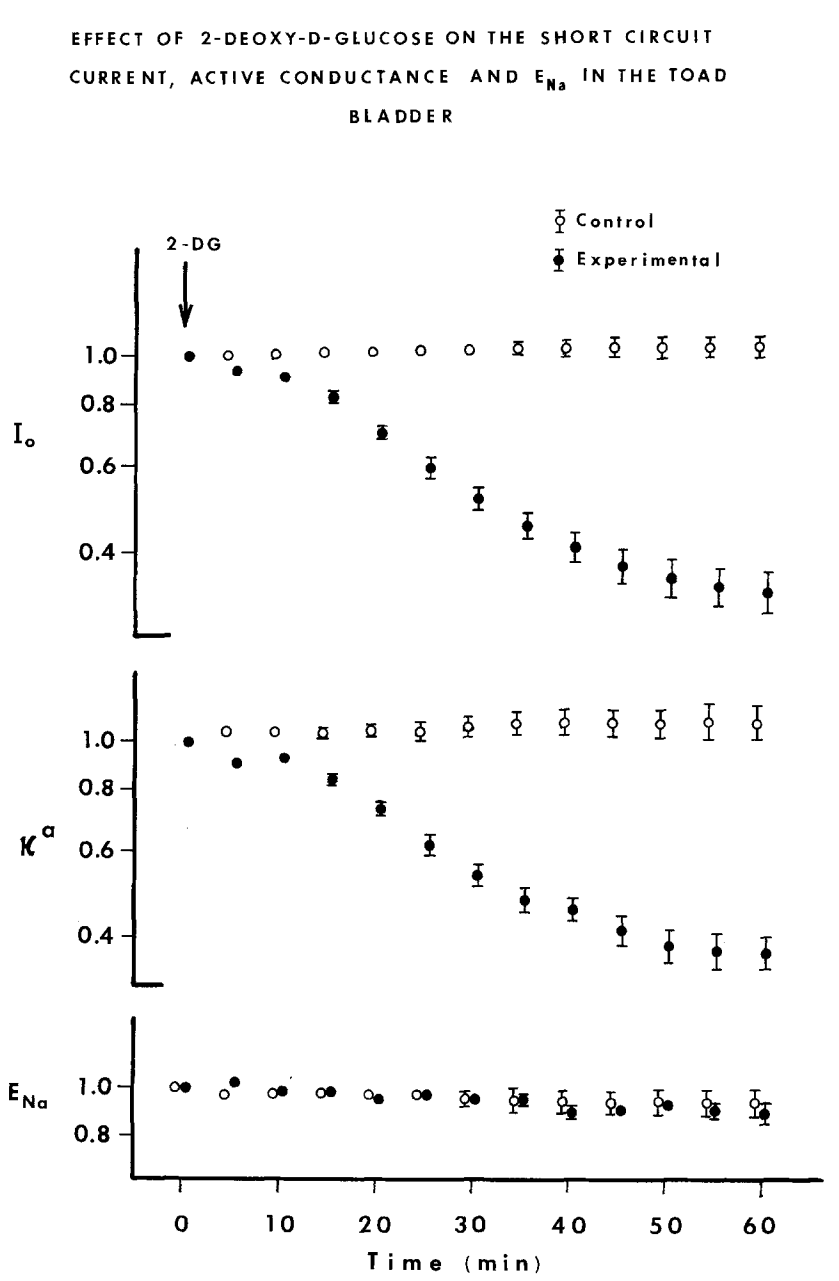


Fig. 2. Effect of 7.5×10^{-3} M 2-deoxy-D-glucose on I_0, κ^a, and E_{Na} (semi-logarithmic plot). All data are normalized to the values at time zero. Actual values (mean $\pm$ SEM, $n=7$) at time zero were:

	I_0 (μA/cm^2)	κ^a (mmho/cm^2)	E_{Na} (mV)
Control	19.2$\pm$3.0	0.191$\pm$0.033	113.7$\pm$9.7
Experimental	19.7$\pm$5.1	0.179$\pm$0.035	121.2$\pm$3.3

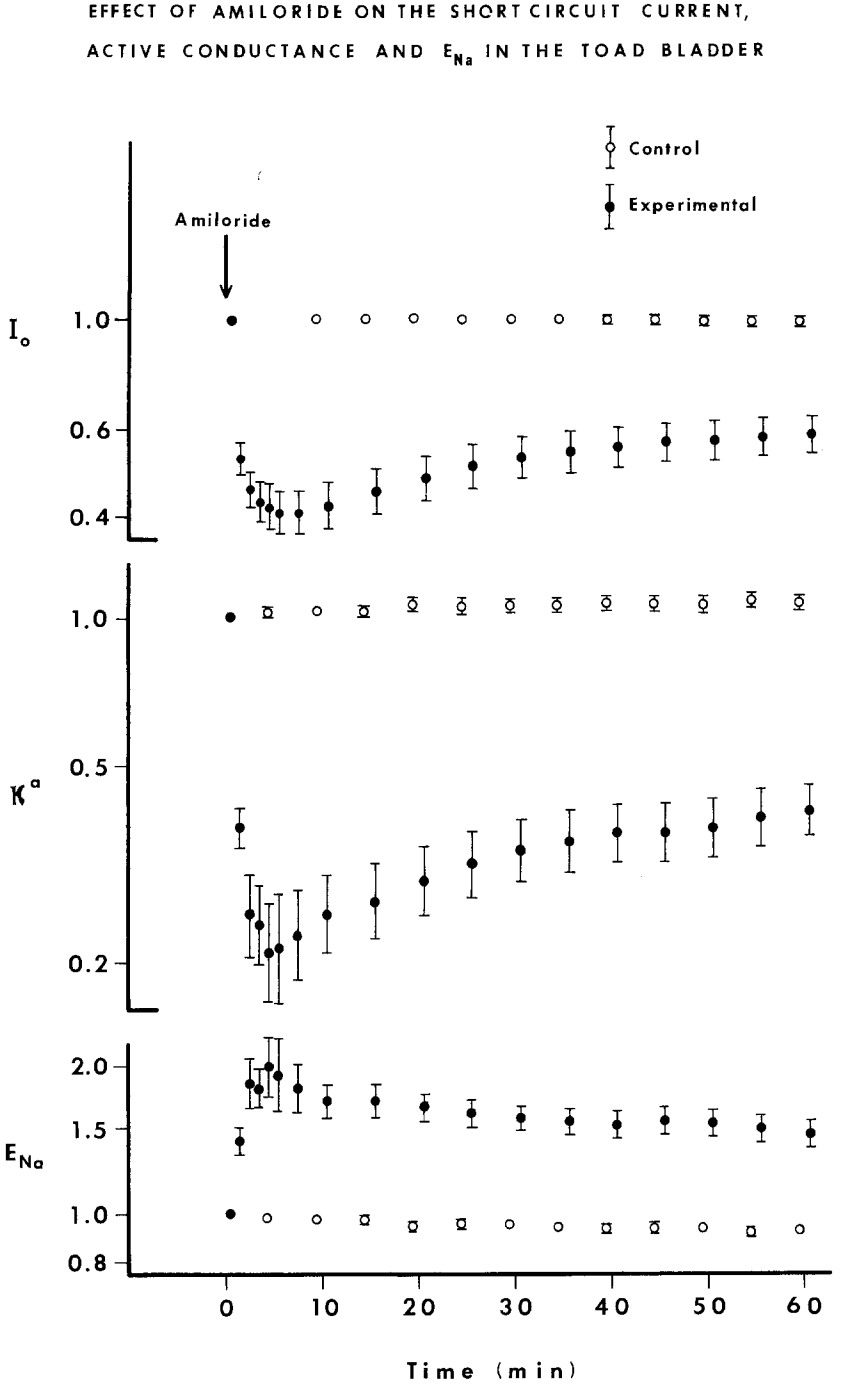


Fig. 3. Effect of 5×10^{-7} M amiloride on I_0, κ^a, and E_{Na} (semi-logarithmic plot). All data are normalized to the values at time zero. Actual values (mean $\pm$ SEM, $n = 6$) at time zero were:

	I_0 (μA/cm^2)	κ^a (mmho/cm^2)	E_{Na} (mV)
Control	27.8 $\pm$ 20.1	0.203 $\pm$ 0.036	127.2 $\pm$ 14.2
Experimental	30.4 $\pm$ 6.2	0.250 $\pm$ 0.059	125.7 $\pm$ 8.4

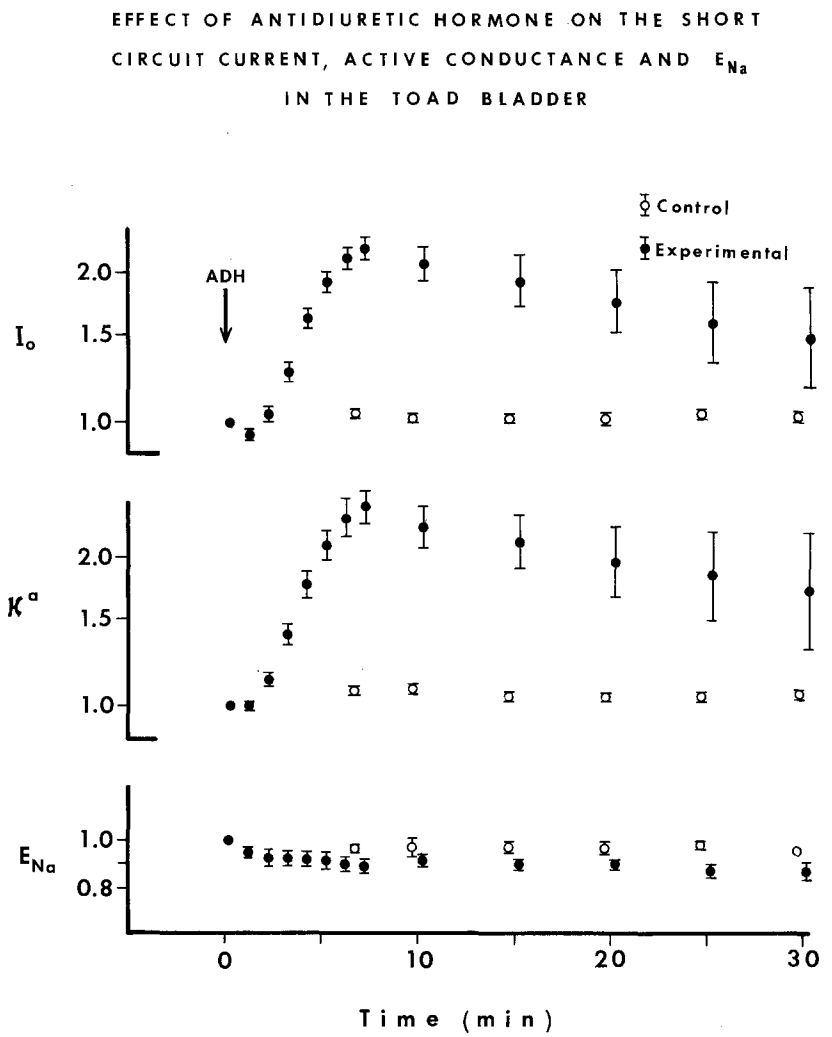


Fig. 4. Effect of antidiuretic hormone (100 mU/ml) on I_0, κ^a, and E_{Na} (semi-logarithmic plot). All data are normalized to the value at time zero. Actual values (mean ± SEM, $n=17$) at time zero were:

	I_0 (μA/cm^2)	κ^a (mmho/cm^2)	E_{Na} (mV)
Control	16.8±3.0	0.146±0.026	118.2±3.7
Experimental	14.7±2.4	0.139±0.024	112.5±5.9

For time $t=0$–7 min, $n=17$; $t=10$–20 min, $n=7$; $t=25$ min, $n=6$; $t=30$ min, $n=5$

determinations of I_0 and κ permit the delineation of the behavior of the equivalent circuit elements with a high degree of temporal resolution. This technique was applied to a detailed study of the effects of 2-deoxy-D-glucose, amiloride, ADH, and ouabain. In the course of the study the effect

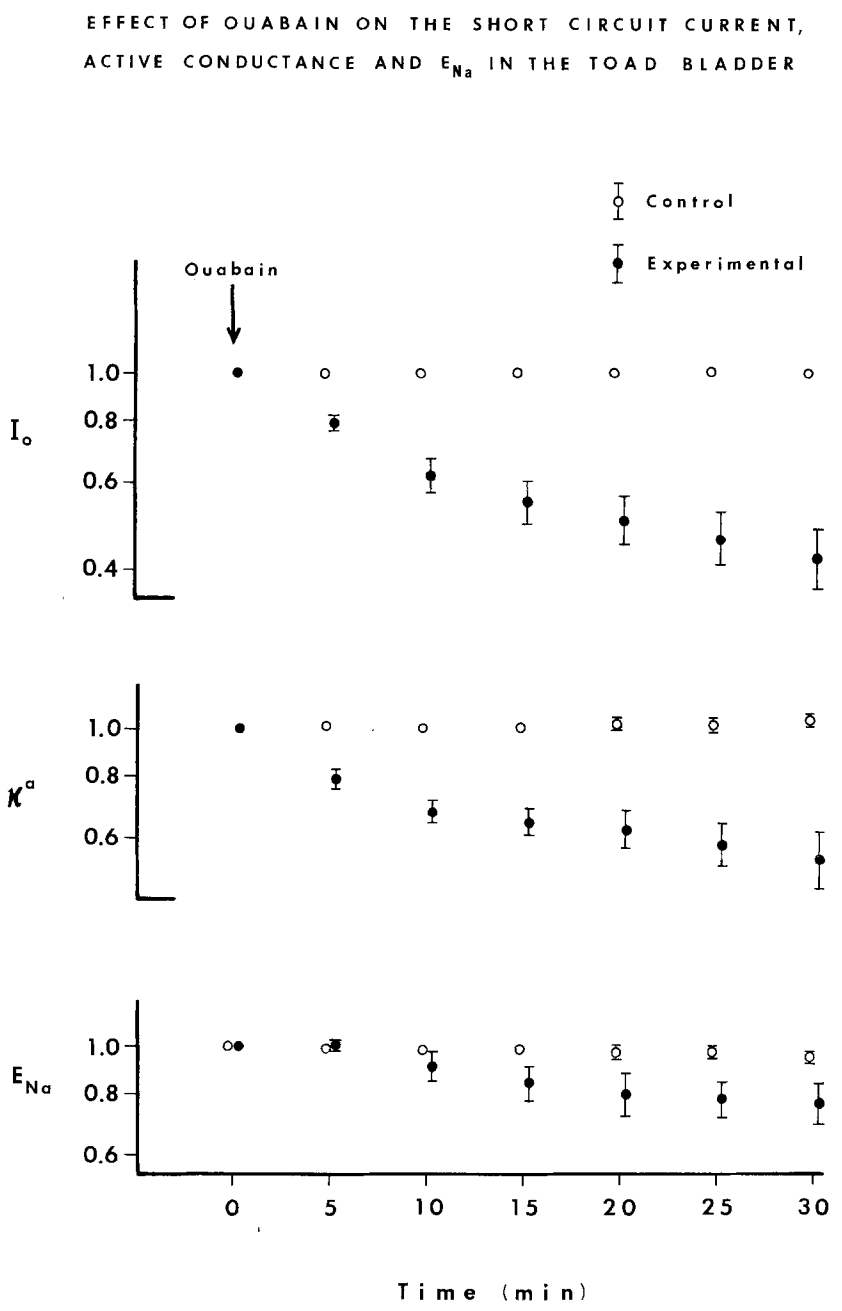


Fig. 5. Effect of 10^{-4} M ouabain on I_0, κ^a, and E_{Na} (semi-logarithmic plot). All data are normalized to the values at time zero. Actual values (mean ± SEM, $n=8$) at time zero were:

	I_0 (μA/cm²)	κ^a (mmho/cm²)	E_{Na} (mV)
Control	24.6±6.4	0.221±0.053	112.8±7.2
Experimental	17.9±2.5	0.153±0.022	119.6±6.3

Table 2. Effects of 2-deoxy-D-glucose, amiloride, antidiuretic hormone, and ouabain on I_0, κ^a, and E_{Na}, as evaluated by the chord intercept technique[a]

Agent	t (min)	n	I_{0t}/I_{00}	κ^a_t/κ^a_0	$E_{Na\,t}/E_{Na\,0}$
2-DG (7.5×10^{-3} M)	60	7	0.33 ± 0.03[1]	0.38 ± 0.03[1]	0.89 ± 0.05
Control	60	7	1.05 ± 0.05	1.11 ± 0.08	0.94 ± 0.05
Amiloride (5×10^{-7} M)	5	6	0.41 ± 0.04[1]	0.21 ± 0.05[1]	1.92 ± 0.33[2]
Control	5	6	1.00 ± 0.01	1.02 ± 0.02	0.98 ± 0.02
ADH (100 mU/ml)	7	17	2.25 ± 0.11[1]	2.53 ± 0.19[1]	0.89 ± 0.03[3]
Control	7	17	1.04 ± 0.01	1.08 ± 0.02	0.97 ± 0.02
Ouabain (10^{-4} M)	30	8	0.41 ± 0.06[1]	0.54 ± 0.07[1]	0.76 ± 0.07[3]
Control	30	8	0.99 ± 0.02	1.04 ± 0.03	0.95 ± 0.03

[a] For 2-DG and ouabain, t was the time at the end of the observation period, when response was maximal. For amiloride and ADH, t was the time for peak response. The data are expressed as in Table 1. Significant difference between control and experimental tissue:

[1] $p<0.001$. [2] $p<0.005$. [3] $p<0.05$.

of each substance on the passive conductance was evaluated by comparing two values of κ^p, one measured 60 min prior to the administration of a test agent and the other at the end of the experiment (both measured with the amiloride-intercept technique). There was no significant difference between the change of κ^p in control tissues and in experimental tissues which received any of the substances employed. It was appropriate, therefore, to calculate values of κ^a and E_{Na} from the value of κ^p at the end of the observation period.

Fig. 2 shows the effects of 7.5 mM 2-deoxy-D-glucose. It is seen that I_0 and κ^a began to decrease promptly after the administration of 2-*DG*, and showed progressive inhibition during the 60 min of observation. However, E_{Na} was unaffected, except for a transient slight increase 5 min after the commencement of treatment.

Fig. 3 shows the effects of long-term exposure to 5×10^{-7} M amiloride. In this case, as for the case of 2-deoxy-D-glucose above, κ^p was determined prior to and following the experimental period by brief exposure to 10^{-5} M amiloride. Following the initial determination of κ^p the 10^{-5} M amiloride was removed by flushing, and after 60 min for restabilization the experimental hemibladder was exposed to 5×10^{-7} M amiloride for 60 min. As is seen, there was prompt and marked inhibition of I_0 and κ^a, and enhancement of E_{Na}. The effects peaked after about 4–5 min, then tapered off, but remained highly significant throughout the 60 min of observation.

The detailed study of the effects of ADH (100 mU/ml) is shown in Fig. 4. After an early transient slight reduction, I_0 increased rapidly, reaching a

peak about 7 min after the administration of ADH. The changes in I_0 were associated with parallel behavior of the conductance of the active pathway, κ^a. Also seen is prompt, slight but significant depression of E_{Na}.

Fig. 5 shows the effects of 10^{-4} M ouabain. I_0 and κ^a were inhibited promptly and progressively. Changes in E_{Na} lagged behind effects on I_0 and κ^a, but at 25 min E_{Na} was significantly below control level.

For comparative purposes the maximal effects of 2-deoxy-D-glucose, amiloride, ADH, and ouabain are summarized in Table 2.

Discussion

Because of the attractive simplicity of the equivalent circuit model of active sodium transport, many have applied it in attempts to distinguish effects on permeability and energetic factors. Various techniques have been used for this purpose [5, 10, 13, 24, 25].

Yonath and Civan's technique offers the advantage of replacing inconvenient substitution or radioactive tracer techniques by the automatic monitoring of electrical parameters, the conductance and short-circuit current. Although it was appreciated that curvature of the $\kappa - I_0$ plots may be observed at the height of the response to vasopressin, it was felt that the inverse slope of linear regions of these plots provided a rapid and reliable technique for the determination of E_{Na} [29]. In our hands curvature of the $\kappa - I_0$ plot was observed regularly on prolonged observation following the administration of either ADH or amiloride. Quite possibly our results with ADH might be the result of the use of a high concentration of ADH (100 mU/ml). Yonath and Civan pointed out an occasional terminal fall in E_{Na} following very high concentrations of vasopressin, which they presumed reflected saturation of the Na^+ pump.

Clearly curvature of the $\kappa - I_0$ plot must invalidate the use of the inverse slope as an accurate means of evaluation of E_{Na}. Less evident is the fact that apparent linearity of the plot does not in itself assure that the reciprocal of the slope represents a unique-valued E_{Na}: constancy of the slope $d\kappa/dI_0$ is compatible with appreciable variation of E_{Na} on perturbation of κ^a and I_0. In such a case, the inverse slope will under-evaluate E_{Na} for $dE_{Na}/dI_0 < 0$ (as with ADH or amiloride) and over-evaluate E_{Na} for $dE_{Na}/dI_0 > 0$ (as with ouabain). In both cases the magnitude of the error will vary of course with time, and may or may not be significant.

Fig. 1 provides an example of a case in which the $\kappa - I_0$ plot was apparently linear during the early response to both stimulation by ADH

and depression by amiloride. Nevertheless, it appeared that the slope of the ADH plot was too steep, since the intercept of its projection was appreciably less than the value of κ^p obtained more directly by the measurement of κ at very low levels of residual short-circuit current (0.127 mmho/cm^2, as compared with 0.149 mmho/cm^2). In this case the value of E_{Na} estimated from the slope technique was 80.5 mV during the ADH period and 71.5 mV during the amiloride period. Successive values calculated by the chord intercept technique were 98.9, 91.4, and 87.7 mV during the ADH period and 89.2, 103, and 166 mV during the amiloride period.

Providing that κ^p can be known, the repeated determination of κ and I_0 and the application of Eq. (5) provide the time course of both κ^a and E_{Na} in fine detail. κ^p could of course be evaluated by tracer isotope techniques, as previously. We have here instead used high doses of amiloride for this purpose. The effect of the amiloride is rapid and reversible. Since amiloride inhibits active sodium transport by depressing κ^a, the values of κ in the absence of active sodium transport (*i.e.* $I_0 = 0$) should represent κ^p. (We presume that in short-circuited toad bladders mounted in chambers I_0 is essentially equivalent to net active sodium transport [12, 14].)

With the persistence of about 5 % of the original short-circuit current following exposure to 10^{-5} M amiloride there was some slight error in our determination of κ^p, which we estimate as about 2–3 % (this error was not demonstrable by the use of radioactive tracer techniques). Since prior to treatment $\kappa^a \simeq \kappa^p$, the error in the determination of initial values of κ^a and E_{Na} from Eqs. (2) and (5) would also have been about 2–3 %. (We assume insignificant error in the measurement of κ and I_0.) With diminution of κ^a this error would become more appreciable. On depression of κ^a to some 20 % of control level following exposure to 5×10^{-7} M amiloride (the lowest levels observed in this study) the over-estimate of κ^a would approximate 10–15 %.

In the present study, in order to accomodate for the possibility of progressive change, κ^p was determined twice, once before and once after exposure to the test agent. In view of the findings that with precaution κ^p may be kept small and near-constant for hours [17], and that a variety of test agents are without effect on κ^p, it may often prove practical to determine κ^p only at the end of an experiment. It should then be possible to use a sufficiently high concentration of amiloride to reduce I_0 to zero and thereby obtain a highly precise value for κ^p, thus eliminating one source of error in the determination of κ^a and E_{Na}.

Since the present technique permits the study of effects on κ^a and E_{Na} with a high degree of temporal resolution, it is of interest to compare our

results with those of others. Values of E_{Na} in control tissues, determined from the chord intercept technique, averaged 117.7 ± 7.2 mV ($n = 37$). This is to be compared with Yonath and Civan's value of 105 ± 2.9 mV ($n = 55$), evaluated by their ADH-slope technique, and Saito, Lief, and Essig's value of 114 ± 13 mV ($n = 28$), based on tracer isotope fluxes [19, 29]. As discussed, we feel that exposure to 100 mU/ml ADH resulted in a slight depression of E_{Na}, but even assuming that the smaller dosages used by Yonath and Civan also depressed E_{Na}, clearly there was no important difference between the control values in their study and in ours.

Very different conclusions were reached concerning the effects of amiloride. Yonath and Civan concluded that exposure to concentrations of $5\text{–}8 \times 10^{-6}$ M for 15 min or longer had no effect on E_{Na}. We considered that exposure to 5×10^{-7} M amiloride dramatically increased E_{Na} as rapidly as we were able to make the measurement, *i.e.* within a minute (Fig. 3), and that this enhancement of E_{Na} was maintained for at least 150 min (Table 1). Larsen found comparably large effects of amiloride on E_{Na} in toad skins bathed in sulfate-Ringer's solution [11]. We are unable to account for the apparent extreme difference in response to amiloride in Yonath and Civan's study and ours. The toads of both laboratories derive from the Dominican Republic; the results of our Tables 1 and 2 represent both techniques employed, and were obtained in different seasons.

In contrast to the findings with amiloride, in both Yonath and Civan's and our study 10^{-4} M ouabain markedly depressed E_{Na}. Again, however, there is a significant difference. Yonath and Civan found that ouabain depressed short-circuit current and E_{Na} proportionately, which according to Eq. (1) would indicate no effect on the active conductance. We found a marked depression of κ^a, with the value after 30 min of exposure being only about half that of paired control tissues.

Of particular interest was the response to the metabolic inhibitor, 2-deoxy-D-glucose. Although this agent depressed I_0 and κ^a promptly and progressively, it had no effect on E_{Na}, other than slight transient early stimulation. These observations raise forcefully the question of the fundamental nature of E_{Na}.

In terms of the classical equivalent circuit model, it seems natural to consider E_{Na}, the electromotive force of sodium transport, to represent the "driving force" for the process of active sodium transport. Indeed it has become customary to analyze the mechanism of action of agents which modify transport by means of this construct. Although our observations of the effects of ADH, amiloride, and ouabain differ somewhat from reports of some others, they are not inconsistent with this point of view. The

effects of 2-deoxy-D-glucose, however, are not consistent with the conventional point of view: the fact that a potent metabolic inhibitor markedly depresses transport without significant depression of E_{Na} surely casts doubt on the role of this parameter as a fundamental energetic determinant. As discussed elsewhere, E_{Na} must reflect both permeability and energetic factors [3, 5, 9].

The nature of this dependence may be made more precise by considering an alternative means of analyzing the energetics of active transport, in terms of the formalism of linear nonequilibrium thermodynamics [9, 18, 26]. In this view, with identical solutions at each surface the rates of active Na transport and the associated suprabasal oxidative metabolism are expressed respectively as

$$J^a_{Na} = L_{Na}(-F\Delta\psi) + L_{Na\,r}A, \tag{6}$$

and

$$J^{sb}_r = L_{Na\,r}(-F\Delta\psi) + L_r A. \tag{7}$$

Here F is the Faraday constant, the Ls are "phenomenological coefficients" incorporating permeabilities and/or rate constants, and A is the affinity (negative free energy) of the oxidative metabolic reaction which is driving active Na transport. Experimental studies in frog skin, toad skin, and toad bladder have supported the validity of Eqs. (6) and (7), and have indicated constancy of the Ls and of A on brief perturbation of $\Delta\psi$ [18, 26]. Accordingly it is possible to relate the parameters of the equivalent circuit and thermodynamic models. Thus,

$$\kappa^a = -(d/d\Delta\psi)(FJ^a_{Na}) = F^2 L_{Na} \quad \text{(constant } A\text{)} \tag{8}$$

and

$$I_0 = (FJ^a_{Na})_{\Delta\psi=0} = FL_{Na\,r}A. \tag{9}$$

Combining Eqs. (1), (8), and (9) shows that

$$E_{Na} = (1/F)(L_{Na\,r}/L_{Na})A. \tag{10}$$

Thus it appears that, in contradistinction to the thermodynamic affinity, the electromotive force of Na transport comprises both permeability and energetic factors.

Studies of the thermodynamic affinity in frog skins have supported the view that A reflects the substrate-product ratio of a metabolic reaction intimately related to the active transport process [8, 16, 18]. In view of fundamental differences between E_{Na} and A on theoretical grounds it is of interest to compare the effects of various agents on these two parameters. Table 3 presents a preliminary comparison based on measurements of

Table 3. Effects of various agents on E_{Na} and the affinity A[a]

Agent	E_{Na}			Source	A			Source
	Dose	t	E_{Na}		Dose	t	A	
2-DG	7.5×10^{-3} M	5–60 min	–	†	1.6×10^{-2} M	1 hr	↓	[16]
Amiloride	5×10^{-7} M	1–60 min	↑	†	$10^{-7} - 10^{-5}$ M	1 hr	–	[18]
					$10^{-7} - 10^{-5}$ M	4 hr	↑	[18]
Ouabain	10^{-4} M	5–30 min	↓	†	10^{-7} M	2.5 hr	–	[16]
Aldosterone	5×10^{-7} M	1–6 hr	–	[17]	5×10^{-7} M	14–18 hr	↑	[18]

[a] E_{Na} was determined in toad bladders. The affinity A was determined in frog skins. Data marked "†" are from the present study. The effect of aldosterone on E_{Na} was studied by tracer isotope techniques [17]. The symbols " – ", "↑", and "↓" represent no change, a significant increase, and a significant decrease respectively.

E_{Na} in the toad bladder and of A in the frog skin. As is seen, three of the agents employed in the present study appear to have discrepant effects on E_{Na} and A. (Present techniques do not permit a determination of A during the transient response to ADH.) In an additional study, 1–6 hr of exposure of toad bladders to aldosterone did not affect E_{Na}, evaluated with tracer isotope techniques [17], while overnight exposure of frog skins markedly enhanced the affinity [18]. It is appreciated that no definitive conclusions can be deduced from studies in different tissues under different experimental conditions. Nevertheless the experimental discrepancies between effects on E_{Na} and A support the inference from the theoretical analysis, that E_{Na} cannot be considered simply an energetic parameter, but must reflect kinetic factors as well.

This is not to say that E_{Na} is without physiological significance. Since E_{Na} is the maximal electrochemical potential difference which can be achieved by the sodium pump, its modulation may be of fundamental importance in the regulation of intracellular sodium activity. Although dramatic changes in E_{Na} were observed in this study, the demonstration that the metabolic inhibitor 2-deoxy-D-glucose rapidly depresses κ^a but not E_{Na} suggests dynamic interaction between permeability and energetic factors so as to protect E_{Na}.

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