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Determination of the Driving Force of the Na^+ Pump in Toad Bladder by Means of Vasopressin

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Summary. Vasopressin stimulates Na^+ transport across toad bladder largely or entirely by decreasing the resistance to Na^+ entry into the transporting epithelial cells. Therefore, the hormone should induce proportional changes in short circuit current (I_S) and tissue conductance; the ratio of these changes should equal the driving force (E_{Na}) of the Na^+ pump.

Administration of vasopressin provided a rapid, reversible and reproducible technique for the measurement of E_{Na}. Values calculated for E_{Na} ranged from 74 to 186 mV, in agreement with previously published estimates. The results were not dependent on the vasopressin concentration over a wide range of concentrations.

Ouabain, an agent thought to inhibit specifically the Na^+ pump, decreased both I_S and E_{Na}. On the other hand, amiloride, a diuretic thought to block specifically Na^+ entry, markedly reduced I_S, without reducing E_{Na}.

It is concluded that vasopressin constitutes a probe for the rapid reproducible determination of E_{Na} under a wide variety of physiological conditions.

The urinary bladder of the toad can produce a net transport of Na^+ from mucosa-to-serosa in the absence of gradients of electrical potential or Na^+ concentration; the net transport may be abolished by metabolic inhibitors [20]. Vasopressin stimulates this active transport of Na^+. On the basis of studies of the radioactive Na^+ pool of whole tissue, Frazier, Dempsey and Leaf [13] postulated that the hormone acted to decrease the passive resistance to Na^+ entry into the transporting epithelial cells. Subsequent investigations have provided support for this hypothesis.

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Civan, Kedem and Leaf [5] demonstrated that when the Na^+ concentration of the mucosal medium is low, vasopressin may reduce the transepithelial resistance without increasing the driving force of the process (Na^+ pump) responsible for the active transepithelial transport of Na^+. Vasopressin must therefore decrease the resistance of a permeability barrier to Na^+ at some site within the tissue.

Determination of the electrical resistance profile across toad bladder before and after vasopressin administration has indicated that vasopressin acts primarily on the apical permeability barrier, with little or no effect on the series basal permeability barrier [4]. Since the submucosa and serosa constitute little resistance to the passage either of electric current [12] or of radioactive tracers [9, 14], and since the mucosal epithelium of toad bladder consists of a single complete layer of cells [7], the apical plasma membrane of the transporting cells is likely to be the site of action of the hormone.

Vasopressin does not reduce the electrical resistance of toad bladder when choline ion replaces Na^+ in the bathing media [3], so that the changes in electrical resistance appear to reflect reliably changes specifically in the resistance to movement of Na^+.

If vasopressin acts to reduce selectively the passive resistance to Na^+ entry into the tissue from the mucosal medium, the hormone should increase the Na^+ concentration within the transporting cells. Studies of the electrolyte composition before and after hormone using whole tissue, including the collagen and smooth muscle of the supporting tissue, have led to conflicting results [13, 17, 18]. However, despite preliminary negative results from studies of isolated epithelia [8], more recent investigations of the electrolyte composition of isolated epithelial cells have clearly demonstrated an increase in Na^+ concentration following vasopressin [22].

This formulation of the mechanism of action of vasopressin has been challenged by an argument based on studies using amphotericin B and low mucosal concentrations of Na^+ [11]. However, other interpretations of the data are possible, and, in a recent study, Bentley [2] has obtained contrary experimental results.

Thus, the evidence indicates that the major or sole action of vasopressin is to decrease the resistance to Na^+ movement through a permeability barrier in series with the Na^+ pump.

It seems very likely that pathways for transepithelial Na^+ movement exist in parallel with the active transport pathway [5, 26]. These parallel leak pathways might reflect a heterogeneity of cell population, the intercellular spaces, or compartmentalization of pathways within a given cell, or may

at least partly reflect damaged tissue. However, the fact that vasopressin increases the radioactive Na^+ flux from mucosa-to-serosa, but not in the reverse direction, suggests that vasopressin acts to reduce the resistance to Na^+ movement solely through the active transport pathway.

To the extent that vasopressin acts only on this resistance element, without changing either the driving force (E_{Na}) of the Na^+ pump or the resistance of parallel leak pathways, the addition of hormone should permit direct measurement of E_{Na}. The conceptual basis for this technique is presented in the following section.

Theory

To a first approximation, the current (I)-voltage (V) relationship of toad bladder is linear over the range of physiologic interest [3, 10, 15], although for large applied fields there are marked deviations from linearity [3, 10]. The magnitude of the intercept with the I-axis represents the short circuit current (I_S), and the intercept with the V-axis represents the spontaneous open circuit potential (V_0) of the tissue.

These properties may be simulated by the simple equivalent circuit of Fig. 1.

Na^+ ions are considered to be actively transported through channels of conductance K_A by a Na^+ pump of driving force E_{Na}. Na^+ as well as other ions cross the tissue through parallel channels of conductance K_L.

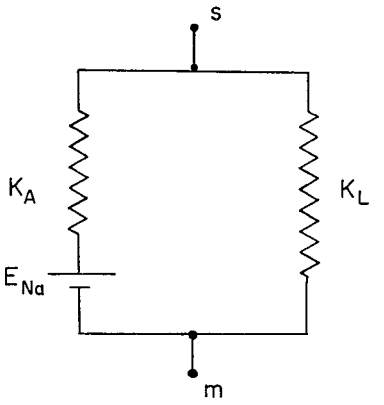

Fig. 1. Simple equivalent circuit of toad bladder. A Na^+ pump of driving force E_{Na} actively transports Na^+ from the mucosal (m) to the serosal (s) medium through a permeability barrier of conductance K_A. Na^+ and other ions may cross the tissue through parallel channels of conductance K_L

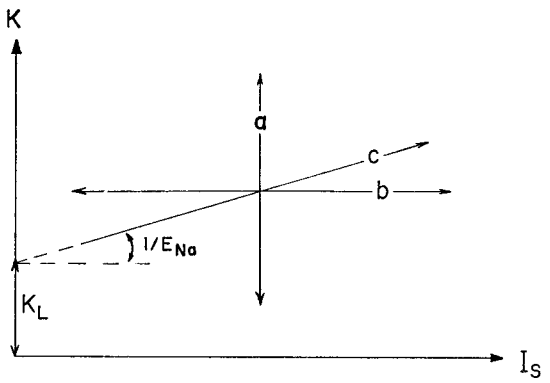

Fig. 2. Fundamental trajectories in the $I_S - K$ plane. The abscissa I_S is the short circuit current; the ordinate K is the total tissue conductance. Each trajectory arises from a change in one of the three circuit elements of Fig. 1. Curves (*a*), (*b*), and (*c*) result from changes solely in K_L, E_{Na}, and K_A, respectively. More complicated trajectories may be produced by processes affecting two or all three circuit elements to varying degrees

The $I-V$ relationship of the circuit is given by:

$$V=\frac{I+E_{Na}K_A}{K_A+K_L}. \tag{1}$$

$V>0$ when the serosal potential exceeds the mucosal potential, and $I>0$ when the flow of positive current is from serosa to mucosa. When the tissue is short-circuited, $V=0$, and the short circuit current I_S is given by:

$$I_S \equiv -(I)_{V=0}=E_{Na}K_A. \tag{2}$$

The total tissue conductivity (K) is given by:

$$K=dI/dV=K_A+K_L. \tag{3}$$

A linear $I-V$ relationship may be characterized by two parameters. We choose for this purpose I_S and K. The electrical properties of the bladder at any given time will be described as a point on the I_S-K plane. Any process which changes the electrical properties of the tissue may be followed by monitoring I_S and K. The time course of these parameters will then trace a trajectory in the I_S-K plane.

In principle, the electrical properties of the tissue may be altered by changing any one of the three circuit elements of Fig. 1. The trajectory traced will be distinctive for each case (Fig. 2), as may be appreciated from Eqs. (2) and (3). (a) A process affecting the leak pathway (K_L) alone will change K but not I_S. (b) A process affecting the driving force (E_{Na}) of the Na^+ pump alone will change I_S but not K. (c) A process affecting the con-

ductance of the active transport pathway (K_A) alone will change both I_S and K. To obtain the analytic expression for the trajectory on the $I_S - K$ plane, K_A is substituted from Eq. (2) into Eq. (3), providing:

$$K = K_L + I_S/E_{Na}. \tag{4}$$

The trajectory is thus a straight line with intercept K_L and slope $1/E_{Na}$.

Processes or agents falling into class (c), but not into classes (a) or (b), may serve as probes for the determination of all three parameters of the equivalent circuit. In particular, this approach provides a measure of the driving force E_{Na} of the Na^+ pump. The technique does not demand that the voltage V be necessarily clamped at 0 mV; similar results may be obtained by fixing V at any other base voltage V_B, and measuring K and the base current I_B arising from Eq. (1):

$$I_B = -(I)_{V=V_B} = (E_{Na} - V_B) K_A - V_B K_L. \tag{5}$$

From Eqs. (3) and (5), the trajectories may now be described on an $I_B - K$ plane. In order to obtain the analytic expression for the trajectory appropriate to processes (c), where only K_A varies, K_A may be substituted from Eq. (5) into Eq. (3):

$$K = \frac{E_{Na} K_L}{E_{Na} - V_B} + \frac{I_B}{E_{Na} - V_B}. \tag{6}$$

Eq. (6) is the more general form of the trajectory equation, reducing to Eq. (4) when $V_B = 0$. Plotted on the $I_B - K$ plane, the slope is now $1/(E_{Na} - V_B)$. For convenience, the reciprocal of the slope will be denoted by E_B:

$$E_B \equiv E_{Na} - V_B. \tag{7}$$

When $V_B = 0$, $E_B = E_{Na}$.

It is clear from Eq. (5) that I_B becomes independent of K_A when $V_B = E_{Na}$. This value I_E will remain constant in the context of processes (c). The $I - V$ relationship of the bladder will therefore be rotated about the point (I_E, E_{Na}) within the $I - V$ plane (Fig. 3). The significance of Eq. (7) is thereby clarified. At a given value of V_B, a process altering K_A alone will in general change the value of I_B; the absolute value of this change ΔI_B will be smaller the closer V_B is to E_{Na}. At $V_B = E_{Na}$, $\Delta I_B = 0$, and ΔI_B will be of opposite sign for $V_B > E_{Na}$ and for $V_B < E_{Na}$.

As noted in the introduction to this paper, vasopressin appears to stimulate active Na^+ transport largely or entirely by increasing K_A (case c), providing a probe for the measurement of E_{Na}. The hormone is particularly convenient insofar as its action is reversible, reproducible, and suitably rapid. The maximal increase in K_A appears some minutes following hor-

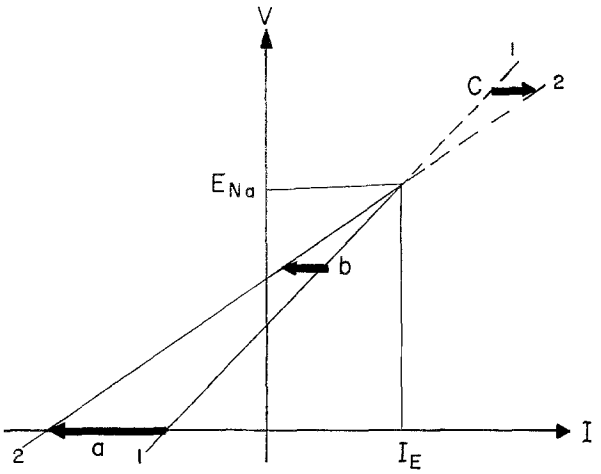

Fig. 3. $I-V$ relationship as a function of K_A. I is the current applied across the tissue in order to maintain the transepithelial potential at V. A process which solely increases K_A, rotates line (1) to line (2). For a given change in K_A, the measured change in $I(\Delta I)$ depends on V [vectors (*a*), (*b*) and (*c*)]. The absolute value of ΔI decreases as V increases from zero to E_{Na} (the driving force of the Na^+ pump); at this point, $\Delta I=0$ (Eq. (5)). As V increasingly exceeds E_{Na}, ΔI increases in magnitude, but is now of opposite sign

monal administration [3], a time course sufficiently slow to permit convenient monitoring by means of a paper chart recorder, yet rapid enough not to be obscured by slower spontaneous time-dependent changes within the tissue.

The following experiments were performed in an effort to (1) determine if administration of vasopressin and application of Eqs. (4) and (6) would provide a reproducible estimate of E_{Na}, (2) compare the calculated value of E_{Na} with previously published estimates of E_{Na}, and (3) measure E_{Na} by this technique under a variety of experimental conditions.

Materials and Methods

Female specimens of the toad, *Bufo marinus*, were obtained from the Dominican Republic (National Reagents, Inc., Bridgeport, Conn.), force fed meal worms upon arrival and weekly thereafter, and maintained on moist wood chips. Urinary hemibladders from doubly pithed toads were mounted in a Lucite double-chamber of 2.6 cm^2 cross-sectional area, providing experimental and control samples from the same tissue [21]. The serosal surface of the tissues was supported by nylon mesh. The chamber volumes were 6 and 5 ml on the mucosal and serosal surfaces, respectively, providing a pressure head of 0.5 cm solution.

The Ringer's solution consisted of (mM): Na^+, 113; K^+, 3.5; Ca^{++}, 0.9; Cl^-, 116; and HCO_3^-, 2.4. The pH was 7.5–8.1, and tonicity was 220 mOsm/kg H_2O.

The experimantal protocol was to clamp the transepithelial potential [23] at the constant values (V_B) and (V_B+10) mV during alternate periods of 10 sec duration. The pulses were provided by an S-4 pulse generator (Grass Instrument Company, Quincy, Mass.). Usually, but not always, V_B was set equal to 0 mV. Potentials were monitored by means of calomel electrodes inserted directly into the bathing media; the presence of a porous disc at the electrode tip reduced diffusion of KCl from the electrode into the bathing media. The current necessary to maintain the transepithelial potential constant was passed through chlorided silver electrodes similarly immersed in the bathing media [3].

Vasopressin (Pitressin, Parke, Davis and Co., Detroit, Mich.) was added to the serosal medium to yield a final concentration usually of 20–40 mU/ml, but occasionally over the range 1–100 mU/ml. In general, the serosal bath was replaced two or three times with fresh Ringer's solution once the peak vasopressin response was reached, in order to permit successive applications of hormone. In certain experiments, 50 λ of 10^{-2} M ouabain (Eli Lilly and Company, Indianapolis, Ind.) was added to the serosal medium, providing a final concentration of 10^{-4} M. Amiloride, obtained as a generous gift from Merck, Sharp and Dohme (Rahway, N.J.), was added in a volume of 50 λ of 10^{-3} M to the mucosal medium of certain preparations, providing a final concentration of 8.3×10^{-6} M.

In several experiments, 1 ml of isotonic Ringer's solution, buffered to pH 8.0, containing 15 mM cyclic 3′, 5′-adenosine monophosphate (cyclic-AMP) was added to a final serosal concentration of 2.5 mM. In order to standardize the technique of drug administration in these experiments, theophylline and vasopressin were also added in volumes of 1 ml isotonic Ringer's solution to final serosal concentrations of 4.2 mM and 6.7 mU/ml, respectively. When a volume of 1 ml was added to the serosal medium, a similar volume of Ringer's solution was introduced into the opposite mucosal medium to maintain the same small gradient of hydrostatic pressure.

Results

Fig. 4a is a representative recording of the transepithelial current when the transepithelial voltage was alternately fixed at 0 and at 10 mV, before and after administration of vasopressin. The upper envelope of the tracing is the short circuit current, and the lower envelope is the current for $V=$ 10 mV. The tissue conductance is then given by:

$$K=\left|\frac{I(10\,\text{mV})-I(0\,\text{mV})}{10}\right|. \tag{8}$$

For any alternation of voltages between V_B and V_B+10 mV, a pair of values may be obtained for (I_B, K) corresponding to the voltage step $V_B \rightarrow V_B+10$ mV or to the step in the reverse sequence. For example, the points $A \rightarrow B$ of Fig. 4b correspond to the transition $10 \rightarrow 0$ mV, and the points $C \rightarrow D$ to the transition $0 \rightarrow 10$ mV.

In certain experiments, these two sets of data pairs constituted a single trajectory in the I_S-K plane, as shown in Fig. 5. In other cases, two distinct trajectories were obtained, as in Fig. 8. In practice, an averaging technique

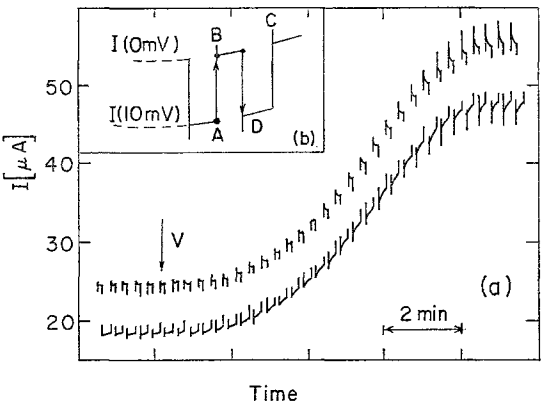

Fig. 4a and b. Measurement of I_S, K. (a) Representative recording of the transepithelial current when the transepithelial potential was alternately fixed at 0 mV (upper envelope) and at 10 mV (lower envelope). The tissue conductance K is then defined as the difference in these two currents divided by the difference in clamping voltage (10 mV). One to two minutes after addition of vasopressin (20 mU/ml) at V, both the tissue conductance and the short circuit current began to increase. The $I_S - K$ trajectory for these data is presented in Fig. 5. (b) Insert defining the method of data reduction. The change in current corresponding to the 10 mV voltage step may be taken to be the excursion $A - B$ (closed circles of Figs. 5 & 7), the excursion $C - D$ (open circles of Figs. 5 & 7), or an average of the two measurements (crosses of Fig. 7). Similarly, the short circuit current (I_S) may be taken to be B, C, or an average of the two measurements. For the present study, the averaging technique formed the basis of data reduction

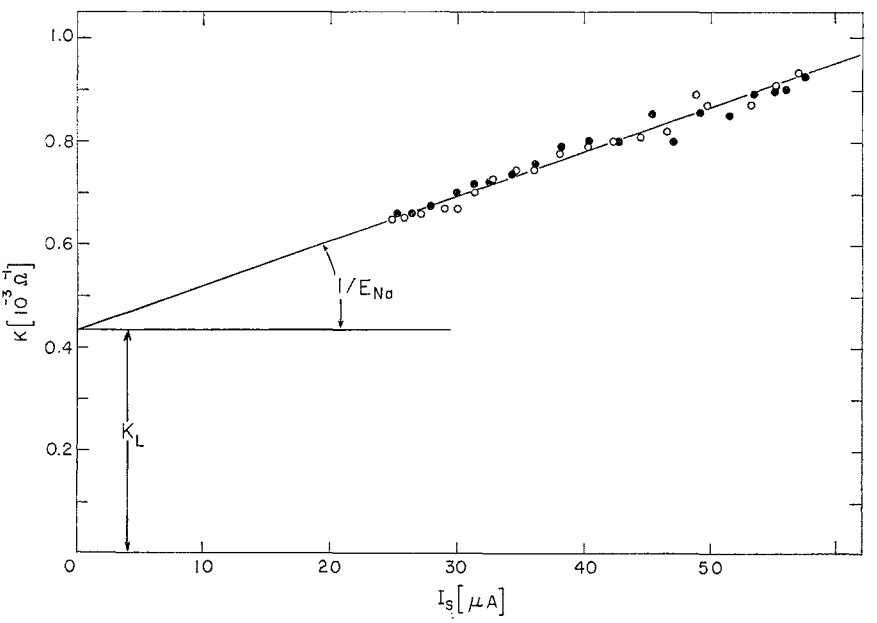

Fig. 5. Representative trajectory in the $I_S - K$ plane, illustrating the method for calculating E_{Na}. The data points have been obtained from the experiment of Fig. 4a. The closed circles are data points obtained from the voltage step $10 \rightarrow 0$ mV, and the open circles from the opposite sequence, $0 \rightarrow 10$ mV. Both series of points may be approximated by the same single straight line; the intercept of this line with the K-axis is K_L, the conductance of the leak pathway. The slope of the line is $(1/E_{Na})$

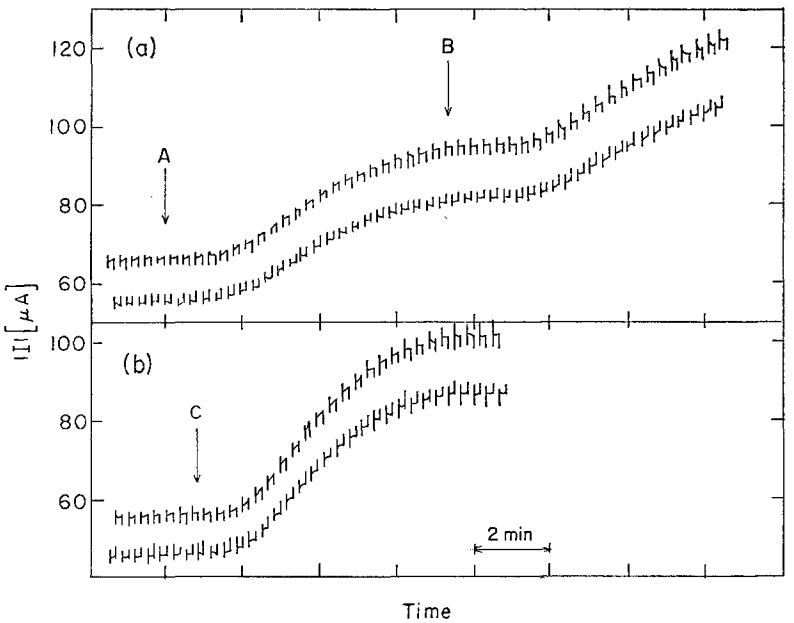

Fig. 6. Effect of concentration on the response to vasopressin. The upper and lower envelopes of the pulses represent the absolute values of the transepithelial current at potential differences of 0 and 10 mV, respectively, as function of time. Vasopressin was added to the serosal medium of one quarter-bladder to a final concentration of 4 mU/ml at time (*A*); the vasopressin concentration was then increased to 40 mU/ml at time (*B*). Vasopressin was added to the serosal medium of the adjoining quarter-bladder to a final concentration of 40 mU/ml in a single step at time (*C*). Data reduction of these records provided the basis for the $I_S - K$ trajectories of Figs. 7 and 8

was utilized to determine the $I_S - K$ relationship; I_S was taken to be the average of points *B* and *C*, and *K* was calculated as the average of the displacements $A - B$ and $C - D$. This averaging technique is illustrated in Figs. 6 and 7, and constituted the basis of the data reduction for all of the recordings.

Precise estimation of *K* was difficult when the current responses to the pulses of constant voltage deviated considerably from square waveforms. Preparations which markedly exhibited this behavior were excluded from the study.

As demonstrated by Fig. 5, *K* was usually a linear function of I_S. In some few experiments, however, the slope of the $I_S - K$ relationship was distinctly greater at the height than at the onset of the response to vasopressin (Fig. 8), consistent with the concept of partial saturation of the Na^+ extrusion step when Na^+ entry was markedly enhanced [20].

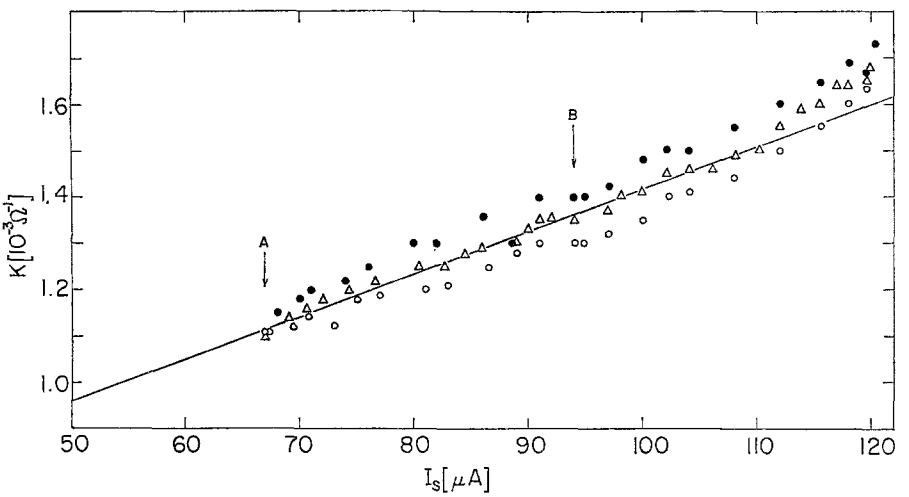

Fig. 7. Effect of method of data reduction on the $I_S - K$ trajectory. The tissue conductance K is plotted as a function of the short circuit current. The data points were obtained from the tracings of Fig. 6a; (*A*) and (*B*) have the same significance as in Fig. 6. The closed circles represent data points obtained for the voltage step $10 \rightarrow 0$ mV; the open circles were obtained for the voltage transition $0 \rightarrow 10$ mV. The triangles were obtained by averaging the results for the two voltage steps, as noted in Fig. 4b; this averaging technique formed the basis of the data reduction for the present study

As long as saturation was not reached, the slope of the $I_S - K$ trajectory, and thus the value of E_{Na} calculated from Eq. (4), was independent of the initial vasopressin concentration (Fig. 9). Similarly, the same value for E_{Na} was obtained by analysis of the $I_S - K$ trajectories produced by successive additive doses of hormone (Fig. 8).

As noted previously, the vasopressin effect was reversible. Replacement of the serosal medium with fresh Ringer's solution two or three times in succession resulted in a new steady state, characterized by values for I_S and K close to the prehormonal levels. Readministration of vasopressin elicited an $I_S - K$ response with a slope close to that of the initial trajectory (Fig. 10a).

Similar values for E_{Na} were also produced by the two halves of mounted hemibladders. Differences noted were of a magnitude similar to those observed for I_S or K, usually not exceeding 20%.

The absolute values calculated for E_{Na} for all 55 quarter-bladders studied under control conditions ranged from 74 to 186 mV (mean $\pm$S.E.M. $= 105 \pm$ 2.9 mV). The total tissue conductance K was usually 0.5 to 1.5 mmho, K_L ranging from 50 to 90% of K at the onset of the experiments. These variations included seasonal changes as well as differences from sample to sample.

In addition to clamping the base voltage at zero, 16 experiments were performed at different values of V_B. As may be noted from Figs. 11 and 12,

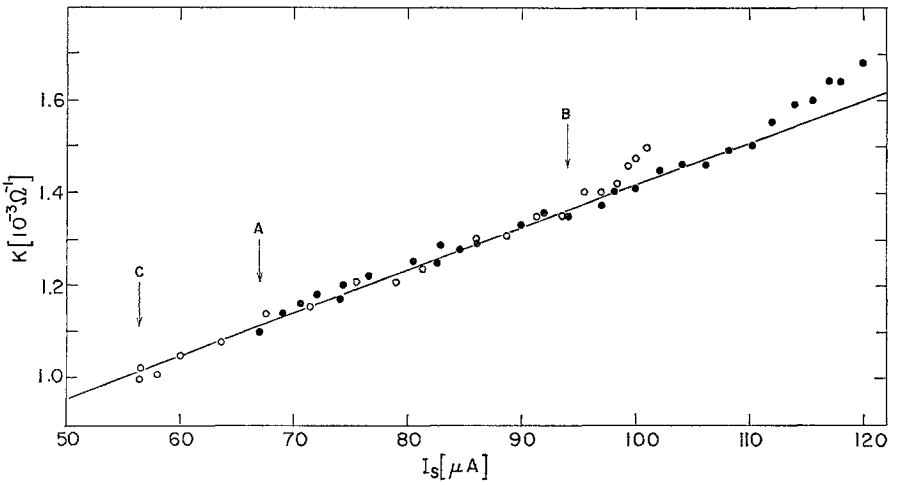

Fig. 8. Effect of concentration on the $I_S - K$ trajectory following vasopressin administration. The tissue conductance K is plotted as a function of the short circuit current by the averaging technique described in Fig. 4b legend for the data of Fig. 6. The closed and the open circles are the data points derived from the records of Figs. 6a and 6b, respectively. The same trajectory is obtained for the two adjoining quarter-bladders. In each case, the response is linear until K has increased by approximately 70%, beyond which the slope increases. Similarly, the response to 4 mU/ml (*A*) falls on the same trajectory traced by the responses to 40 mU/ml (*B, C*). The increase in slope near the peak of the vasopressin response was occasionally seen, consistent with the concept of partial saturation of the Na^+ pump

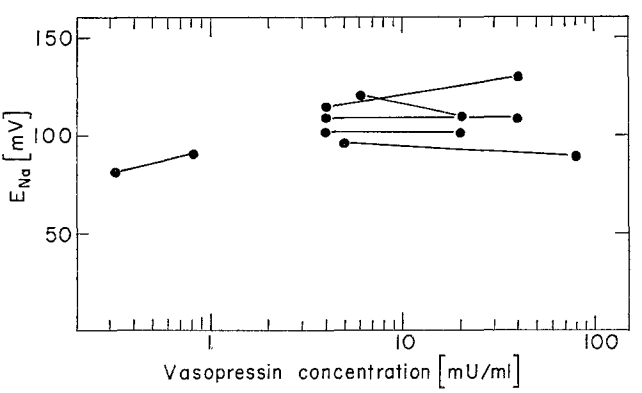

Fig. 9. Effect of vasopressin concentration on E_{Na}. Each pair of connected points was obtained by simultaneously administering two different concentrations of vasopressin to the two adjoining halves of a different hemibladder. E_{Na} was calculated from the resulting $I_S - K$ relationship according to Eq. (4). It is clear that E_{Na} was insensitive to hormonal concentration over the wide range of concentration employed

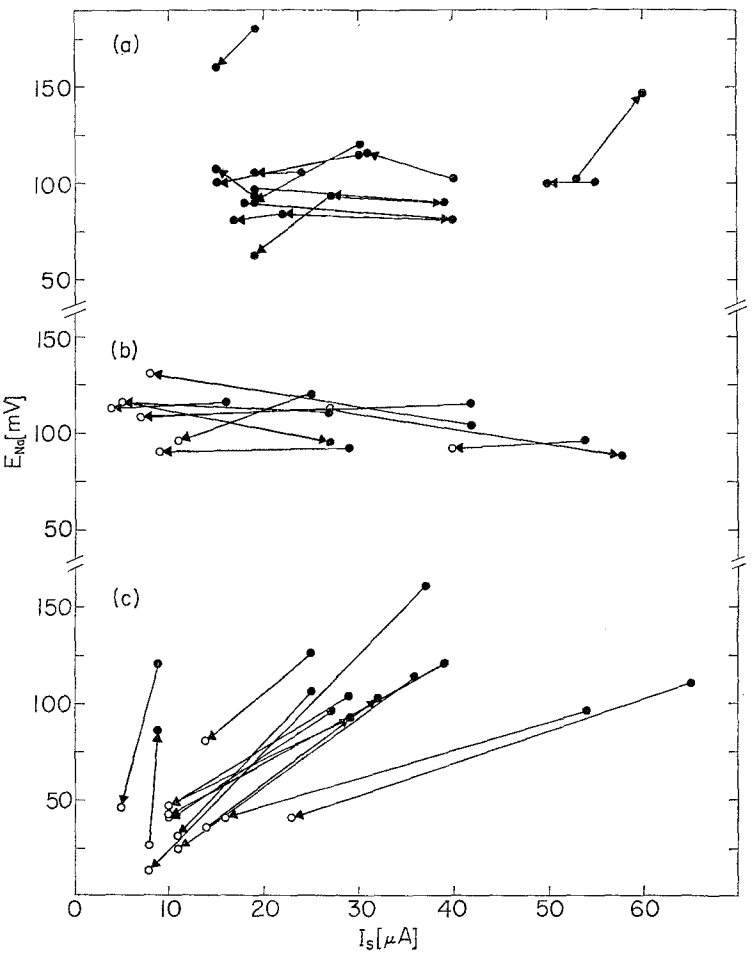

Fig. 10a–c. Effect of amiloride and ouabain on E_{Na}. E_{Na} was calculated from Eq. (6) and plotted as a function of the short circuit current. The closed circles were derived from tissues bathed by Ringer's solution alone; the open circles were derived from preparations treated either with 0.8×10^{-5} M amiloride (b) or 10^{-4} M ouabain (c). Immediately after E_{Na} was determined by means of vasopressin, the serosal medium was replaced two or three times with fresh Ringer's solution; if amiloride was present, the mucosal medium was also replaced two or three times with fresh solution. Successive points were separated by periods of time ranging from 45 to 130 min, during which amiloride or ouabain was either added or washed out. The data of this figure form the basis for the statistical analysis of the Table. (a) *Controls:* successive applications of vasopressin resulted in no appreciable change in E_{Na}. (b) *Amiloride:* the diuretic did not decrease E_{Na}, despite a marked inhibition of the short circuit current. (c) *Ouabain:* under the conditions of the experiment, ouabain produced an approximately proportional reduction in short circuit current and E_{Na}

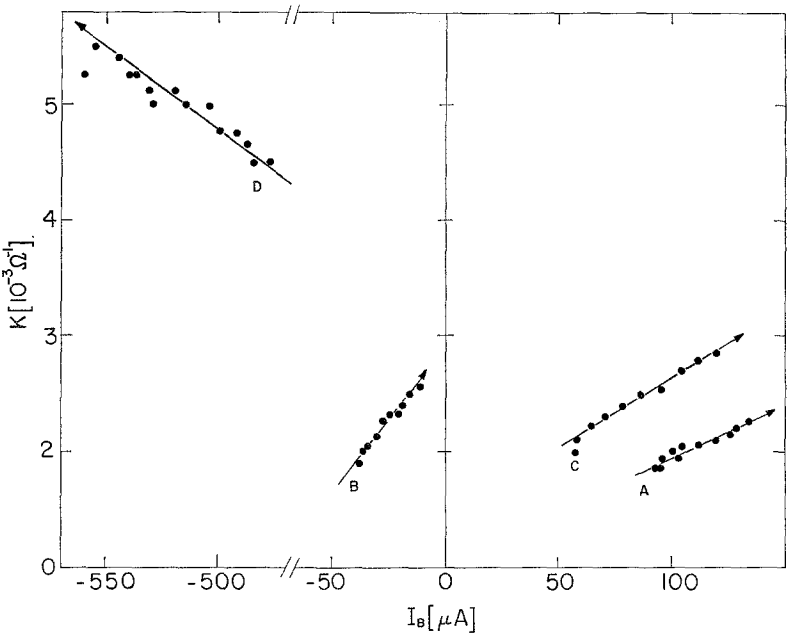

Fig. 11. *A–D*. Effect of the clamping voltage (V_B) on E_{Na}. Vasopressin was successively administered to the same quarter-bladder to obtain trajectories *A–D*. Each treatment was followed by replacement of the serosal medium two or three times with fresh Ringer's solution. (*A*) Short circuited preparation: $V_B=0$ mV, $E_B=110$ mV. (*B*) Preparation clamped at potential greater than the open circuit potential: $V_B=70$ mV, $E_B=40$ mV. (*C*) Short circuited preparation: $V_B=0$ mV, $E_B=80$ mV. (*D*) Preparation clamped at potential exceeding E_{Na}: $V_B=140$ mV, $E_B=-70$ mV

the slope of the I_S-K relationship did increase, as V_B was increased, in qualitative agreement with Eq. (6). Precise data were difficult to obtain at high values because of bladder instabilities; however, in one successful experiment (Figs. 11 & 12), the slope of the I_B-K relationship was actually negative [*see* vector (c) of Fig. 3] for sufficiently high values of V_B, as predicted by Eq. (6).

In view of the strong experimental evidence favoring the concept that cyclic-AMP is at least one, if not the sole, intracellular mediator of vasopressin [24], it was of interest to compare the I_S-K trajectories produced by the two agents. Addition of cyclic-AMP to a final serosal concentration of 2.5 mM in four preparations caused an increase in conductance and short circuit current, and a linear I_S-K trajectory. The subsequent addition of vasopressin to the same quarter-bladder to a final serosal concentration of 6.7 mU/ml produced an I_S-K trajectory which fell on the same straight

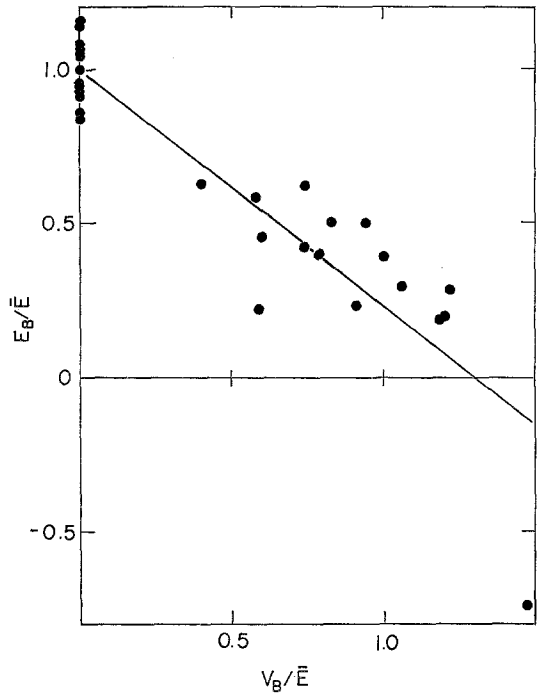

Fig. 12. E_B as a function of V_B. Data were obtained from 16 quarter-bladders. In order to normalize the data, both V_B and E_B [Eq. (6)] were divided by the factor $\bar{E}$; $\bar{E}$ was defined for each quarter-bladder as the average value of E_{Na}, calculated according to Eq. (4), when V_B was set equal to zero (short circuited preparation). The point $V_B/\bar{E}=0$, $E_B/\bar{E}=1$ represents 10 measurements. The equation of the line drawn, calculated by the method of least squares, is: $(E_B/\bar{E})=(1.00)-(0.77\pm0.051)\,(V_B/\bar{E})$, consistent with the concept [Eq. (6)] that E_B is inversely dependent on V_B. The slope is significantly different from one, as noted in the *Discussion*

line (Fig. 13). In four additional experiments, theophylline and vasopressin were sequentially administered to the same preparation; in each case, the trajectories elicited fell on the same straight line (Fig. 13). Thus, administration of vasopressin, cyclic-AMP, and theophylline provided the same estimate of E_{Na} in each of the quarter-bladders studied.

In order to examine further the relationship between the slope of the I_S-K relationship and active Na^+ transport, ouabain was added to 13 preparations. As demonstrated in Fig. 14, ouabain reversibly decreased E_{Na}, calculated from the slope of the I_S-K trajectories, obtained by adding vasopressin to the ouabain-treated and untreated preparations. The glycoside reduced I_S and E_{Na} proportionately (Fig. 10c; Table), consistent with the current concept that ouabain is a specific inhibitor of active Na^+ transport [16].

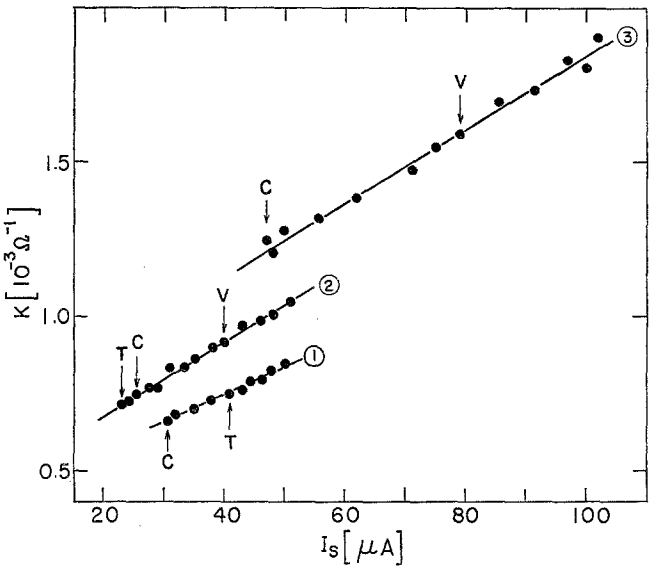

Fig. 13. Effects of cyclic 3′,5′-adenosine monophosphate (cyclic-AMP), theophylline, and vasopressin on the $I_S - K$ relationship. (1) Cyclic-AMP (C) was added to a final serosal concentration of 2.5 mM, following which theophylline (T) was added to the same preparation to a final concentration of 4.2 mM. The trajectories produced by both agents lie on the same straight lines ($E_{Na} = 110$ mV). (2) Theophylline (T) was added to a final serosal concentration of 4.2 mM, producing a modest response. Subsequently, cyclic-AMP (C) and vasopressin (V) were added to the same preparation to final serosal concentrations of 2.5 mM and 6.7 mU/ml, respectively. The trajectories resulting from the three agents lie on the same line ($E_{Na} = 84$ mV). (3) Cyclic-AMP (C) was added to a final serosal concentration of 2.5 mM, following which vasopressin (V) was added to a final serosal concentration of 6.7 mU/ml. The two resulting trajectories fall along the same straight line ($E_{Na} = 85$ mV)

Table. *Effect of amiloride and ouabain on* E_{Na}

Treatment	E'_{Na}/E_{Na}	I'_S/I_S	$(E'_{Na}/E_{Na})/(I'_S/I_S)$
Controls [31]	0.98 ± 0.049	0.95 ± 0.14	1.22 ± 0.11
	($P > 0.7$)	($P > 0.7$)	($P > 0.05$)
Amiloride [9]	1.04 ± 0.053	0.33 ± 0.064	4.1 ± 0.67
	($P > 0.4$)	($P < 0.001$)	($P < 0.01$)
Ouabain [14]	0.36 ± 0.038	0.42 ± 0.049	0.93 ± 0.11
	($P < 0.001$)	($P < 0.001$)	($P > 0.5$)

The values for the driving force (E_{Na}) of the Na^+ pump and the short circuit current (I_S) were obtained from the data of Fig. 10. The primed quantities either represent measurements made 45–130 min after measurement of the initial unprimed values (controls), or represent measurements performed in the presence of an inhibitor (amiloride or ouabain) after the same period of time. The numbers are the means $\pm$ S.E.M. P is the probability that the mean is insignificantly different from one.

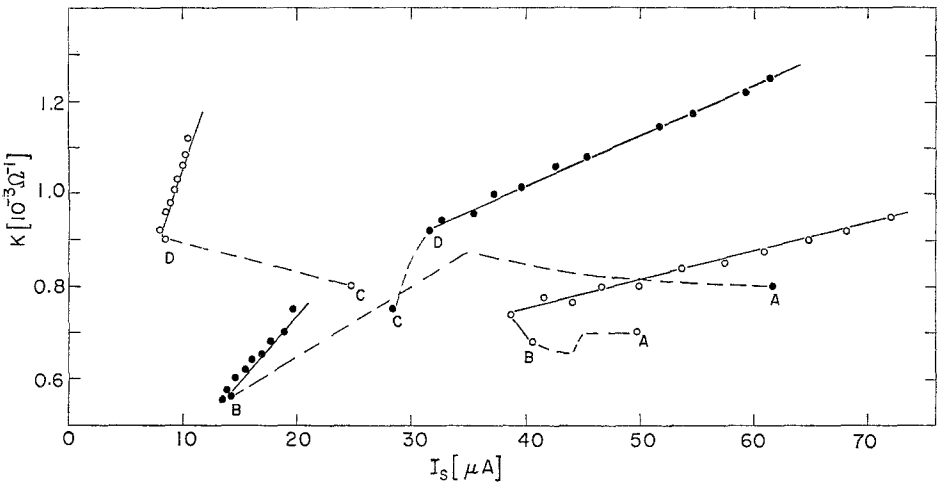

Fig. 14. Effect of ouabain on the $I_S - K$ trajectory produced by vasopressin. The closed and open circles are data points obtained from adjoining halves of a single hemibladder. Data points (*A*) were measured 20 min after mounting the tissue. Ouabain was then added to a final serosal concentration of 10^{-4} M on one side of the preparation (closed circles). Over the ensuing 45 min, the tissues traced the trajectories indicated by the interrupted lines to points (*B*), at which time vasopressin was added to both sides to a final serosal concentration of 20 mU/ml. From the slope of the subsequent $I_S - K$ trajectory, E_{Na} was calculated by Eq. (4) to be 35 and 162 mV for the ouabain-treated and control preparations, respectively. The serosal media of both quarter-bladders were then twice replaced with fresh Ringer's solution; 35 min later, data points (*C*) were measured, at which time ouabain was added to a final serosal concentration of 10^{-4} M on the formerly control side (open circles). At points (*D*), 35 min after (*C*), vasopressin was added to both serosal media to a final concentration of 40 mU/ml; from the resulting trajectories, E_{Na} was calculated to be 95 mV (closed circles) and 13 mV (open circles), respectively. Ouabain therefore reversibly reduced the magnitude not only of the short circuit current, but also of E_{Na}

In order to determine if the fall in E_{Na} might not have been a non-specific result of depression of active transport, the effect of amiloride was studied in nine preparations. Because of the considerably greater rapidity of action of this diuretic, in comparison to either ouabain or vasopressin, the trajectory was usually determined only from the initial and final values of I_S and K.

As demonstrated in Fig. 15, amiloride and ouabain have very different effects on E_{Na}. Amiloride was added to the mucosal medium of one half, and ouabain to the serosal medium of the adjoining half of the preparation. Although the two inhibitors caused similar reductions of I_S on the two sides, amiloride did not measurably alter E_{Na}, whereas ouabain caused a marked fall in E_{Na} to less than half of its initial value.

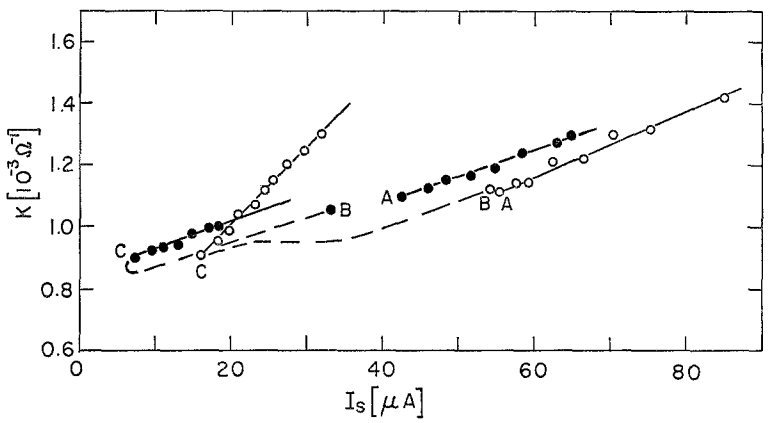

Fig. 15. Effects of amiloride and ouabain on the $I_S - K$ trajectory produced by vasopressin. Data points (*A*) were measured 80 min after mounting, following which vasopressin was added to the serosal media (16 mU/ml). E_{Na} was calculated to be 115 mV (closed circles) and 96 mV (open circles). At points (*B*), 30 min after replacing the serosal media three times with fresh Ringer's solution, amiloride was added to the mucosal medium (5×10^{-6} M) of one side (closed circles) and ouabain added to the serosal medium (10^{-4} M) of the adjoining quarter-bladder (open circles). Over the subsequent 15 min, amiloride reduced the short circuit current and conductance to point (*C*) (closed circle). Ouabain caused a similar reduction in short circuit current over a period of 27 min to point (*C*) (open circle). At points (*C*), vasopressin was again added to the serosal media of both preparations to a final concentration of 100 mU/ml. From the resulting trajectories, E_{Na} was calculated to be 108 mV following treatment with amiloride (closed circles), and 40 mV following treatment with ouabain (open circles)

A summary of the effects of amiloride on E_{Na} is presented in Fig. 10b and the Table. Comparing the changes following diuretic with the changes after ouabain (Fig. 10c) or spontaneously with time (Fig. 10a), it is clear that E_{Na} was not reduced by amiloride under the conditions of the present study.

Discussion

Na^+ transport across toad bladder is thought to proceed first by Na^+ entry from the mucosal medium into the cell in accordance with the electrochemical gradient for Na^+ across the apical cell membrane, followed by the extrusion of Na^+ from the cell into the serosal medium by a Na^+ pump. For the concept of a Na^+ pump to be operationally meaningful, the driving force (E_{Na}) of the Na^+ pump should be measurable. The technique presented permits ready calculation of E_{Na} from measurements of the changes in short circuit current and transepithelial conductance induced by vasopressin. To the extent that vasopressin not only reduces the resistance to Na^+ movement through the active transport channels, but also reduces the

resistance to Na^+ movement through parallel leak channels, the calculated E_{Na} will provide a falsely low estimate of the true value; to the extent that vasopressin also may stimulate the Na^+ pump, the calculated E_{Na} will be a falsely high measure of the true E_{Na}.

It seems likely that for at least four reasons, however, the technique does in fact present a realistic estimated value of E_{Na}. First, as noted in the introduction to this paper, the evidence thus far presented in the literature supports the hypothesis that vasopressin acts primarily or solely to reduce the resistance to Na^+ entry into the tissue. Consistent with this concept, the $I_S - K$ trajectories produced by vasopressin were linear.

Second, under a variety of experimental circumstances, the E_{Na} calculated by the present technique is changed in the same direction predicted on the basis of other independent techniques. When the short circuit current was reduced by ouabain, an agent thought to specifically block active Na^+ transport in toad bladder [16], E_{Na} fell. When the short circuit current was reduced by amiloride, an agent thought to inhibit Na^+ entry [1], no fall in E_{Na} was noted. On the other hand, when the short circuit current was increased by increasing concentrations of vasopressin, no increase in E_{Na} was seen; on the contrary, a terminal fall in E_{Na} was occasionally observed following very high concentrations of vasopressin, presumably reflecting saturation of the Na^+ pump.

Third, as predicted by Eq. (6), the slope of the $I_B - K$ trajectory was directly dependent upon the clamping voltage V_B. It is of interest, however, that the slope of the $V_B - E_B$ relationship (Fig. 12) deviated significantly from (-1). Such an effect might arise from at least two factors. (a) E_{Na} may be inversely dependent on the rate of active transport of Na^+. For example, the efficiency of coupling between the metabolic and transport rates may be increased by reducing the rate of transport work. (b) There may be a heterogeneity of values of E_{Na} among the transporting cells of the epithelium. It is likely that there is a greater resistance to Na^+ movement from serosa to mucosa through the active channels than in the usual physiologic direction [3]. To the extent that V_B exceeds the value of E_{Na} of some of the cells, the cells with lower values of E_{Na} should provide a considerably smaller contribution to the average E_{Na} measured for the entire preparation. As V_B is increased, therefore, there may be an increasingly marked selection for the highest values of E_{Na} among the total population of cells.

Fourth, the mean value of E_{Na} is in reasonable agreement with previous independent estimates. On the basis of studies of the effect of vasopressin on tissue bathed with mucosal solutions containing low concentrations of Na^+, E_{Na} has been calculated to be some 170 mV [5].

Analysis of the current-voltage relationship of toad bladder has similarly suggested that $E_{Na} = 172$ to 184 mV [3]. However, vasopressin was also noted to increase the transepithelial current I_E when V was equal to the presumed value of E_{Na}. From the considerations presented in the *Theory* section, it is likely that the estimate of E_{Na} from that study was somewhat in excess of the true value.

The estimates of E_{Na} obtained by Ussing [27] are also not far different from that of the present study, but both his and Linderholm's [21] results must be interpreted with caution [19] since they are based on an explicitly assumed but improbable homogeneity of transport pathways and an assumed absence of isotope interactions in the radioactive data from which the estimates were taken.

The action of vasopressin is thought to arise from stimulation of adenyl cyclase activity, increasing the conversion of ATP to cyclic-AMP [24]. Therefore, administration of: (1) cyclic-AMP directly; (2) vasopressin, a stimulant of cyclic-AMP production; or (3) theophylline, an inhibitor of the rate of hydrolysis of cyclic-AMP, should all induce similar changes in the transport and electrical characteristics of the tissue. However, Cuthbert and Painter [6] have observed that vasopressin and theophylline increase the tissue conductance, whereas cyclic-AMP decreases the tissue conductance of frog skin; partly on the basis of this observation, they have questioned the intermediary role of cyclic-AMP in the action of vasopressin. This observation was not confirmed in the present study of toad bladder. Vasopressin, cyclic-AMP, and theophylline all increased the tissue conductance, and elicited similar $I_S - K$ trajectories, as anticipated from current concepts of the mode of action of vasopressin.

We conclude that the technique presented is self-consistent, and suggest that this approach may permit ready definition of the mechanism of agents affecting active Na^+ transport across toad bladder.

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