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Relation between Intracellular Sodium and Active Sodium Transport in Rabbit Colon: Current-Voltage Relations of the Apical Sodium Entry Mechanism in the Presence of Varying Luminal Sodium Concentrations

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Summary. The current-voltage relations of the amiloride-sensitive Na entry pathway across the apical membrane of rabbit descending colon, exposed to a high K serosal solution, were determined in the presence of varying mucosal Na activities, $(Na)_m$, ranging from 6.2 to 99.4 mM. These relations could be closely fit to the "constant field" flux equation yielding estimates of the permeability of the apical membrane to Na, P_{Na}^m, and the intracellular Na activity, $(Na)_c$. The following empirical relations emerged: (i) $(Na)_c$ increased hyperbolically with increasing $(Na)_m$; (ii) P_{Na}^m decreased hyperbolically with increasing $(Na)_m$ and linearly with increasing $(Na)_c$; (iii) spontaneous variations in Na entry rate at constant $(Na)_m$ could be attributed entirely to parallel, spontaneous variations in P_{Na}^m; (iv) the rate of Na entry increased hyperbolically with increasing $(Na)_m$ obeying simple Michaelis-Menten kinetics; (v) the relation between $(Na)_c$ and "pump rate," however, was sharply sigmoidal and could be fit by the Hill equation assuming strong cooperative interactions between Na and multiple sites on the pump; the Hill coefficient was 2–3 and the value of $(Na)_c$ at which the pump-rate is half-maximal was 24 mM. The results provide an internally consistent set of relations among Na entry across the apical membrane, the intracellular Na activity and basolateral pump rate that is also consistent with data previously reported for this and other Na-absorbing epithelia.

Key Words rabbit descending colon · electrophysiology · current-voltage relations · sodium transport · cell Na activity

Introduction

The relations among the Na activity in the mucosal solution, $(Na)_m$, the intracellular Na activity, $(Na)_c$, and the activity of the basolateral pump mechanism in "tight" or "moderately tight" amiloride-sensitive Na-absorbing epithelia has been explored in relatively few instances (Li, Palmer Edelman & Lindemann, 1982; Palmer, Li, Lindeman & Edelman, 1982; Thomas, Suzuki, Thompson & Schultz, 1983) and remains unclear. The purpose of this study was to examine these relations for the case of rabbit descending colon, a "moderately tight" epithelium that closely resembles amphibian skin and urinary bladder and rabbit urinary bladder with respect to transcellular Na absorption (Schultz 1981*a*; Thompson, Suzuki & Schultz, 1982*a*).

Although $(Na)_c$ can be determined using ion-selective microelectrodes, this approach is fraught with experimental difficulties which include extremely high tip-resistances, which can lead to shunting of the signal through the glass capillary wall (Lewis & Wills, 1980), and the need to simultaneously determine the electrical potential difference across the apical membrane (ψ^{mc}) with conventional (KCl-filled) microelectrodes preferably in the same cell. In the present study an alternative approach was employed using the highly selective apical Na entry mechanism *itself* as an electrode to measure $(Na)_c$. This approach is based on the observation that Na entry across the apical membrane of rabbit descending colonic cells conforms to the Goldman-Hodgkin-Katz (GHK) "constant-field" flux equation for a single cation over a reasonably wide range (Thompson et al., 1982*a*) as is the case for frog skin (Fuchs, Larsen & Lindemann, 1977), toad urinary bladder (Palmer, Edelman & Lindemann, 1980; Li et al., 1982; Palmer et al., 1982) and *Necturus* urinary bladder (Frömter, Higgins & Gebler 1981; Schultz, 1981*a*; Thomas et al., 1983). Consequently, fitting the parameters of the GHK equation to the "instantaneous" relation between the Na current across the apical membrane (I_{Na}^m) and the electrical potential difference across that barrier (ψ^{mc}) yields an estimate of $(Na)_c$ as well as an estimate of the permeability of the apical membrane to Na, P_{Na}^m.[1]

The methods employed in these studies to determine the relation between I_{Na}^m and ψ^{mc} in the

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[1] Although the ranges over which the GHK equation satisfied the data varied in these studies, in all instances there was an excellent fit over the range $\psi^{mc}=0$ to $\psi^{mc}=E_{Na}^m$. In any event, if Na entry is strictly diffusional, $(Na)_c$ can always be determined from the "reversal (equilibrium) potential" and, knowing $(Na)_c$, P_{Na}^m can then be determined from the relation $I_{Na}^m = \mathcal{F} P_{Na}^m [(Na)_m - (Na)_c]$ when $\psi^{mc}=0$. Thus, strict conformity with the GHK formalism is not necessary.

presence of varying $(Na)_m$ are essentially those described by Thompson et al. (1982*a*). In addition, all studies were carried out in the presence of a high-K serosal solution, an approach originally introduced by Fuchs et al. (1977). The rationale behind this approach is that to the extent that the basolateral membrane approximates the behavior of a K-electrode (particularly in the presence of an impermeant anion) increasing the K activity in the serosal solution to a level that approximates that in the cell should depolarize that barrier and markedly reduce its resistance so that the electrical properties of the epithelial cell are dominated by those of the apical membrane. Thus, in the presence of a high-K serosal solution, one can voltage-clamp the apical membrane itself so that the difference between the transepithelial current at any clamping potential before and after blocking the Na entry step by the addition of amiloride to the mucosal solution is a direct measure of the Na current across the apical membrane at that clamping potential. Thompson et al. (1982*a*) have provided direct evidence validating this approach for the case of rabbit descending colon. Briefly, these investigators found that in the presence of a high-K serosal solution the basolateral membrane resistance fell and the electrical potential difference across that barrier was essentially zero so that the transcellular electrical potential difference, ψ^{ms}, did not differ significantly from that across the apical membrane alone (ψ^{mc}). These findings are entirely consistent with those reported by Wills, Lewis and Eaton (1979), who found that the permeability of the basolateral membrane of rabbit descending colonic cells is dominated by the permeability of that barrier to K.

Symbols and Abbreviations

ψ^{ms}	transepithelial electrical potential difference with reference to the mucosal solution (mV)
ψ^{mc}	electrical potential difference across the apical membrane with reference to the mucosal solution (mV); under the conditions of the present experiments (depolarization of the basolateral membrane by the high-K serosal solution) $\psi^{mc} \approx \psi^{ms}$
I_{Na}	Na current ($\mu A/cm^2$)
I^m_{Na}	Na current across the apical membrane ($\mu A/cm^2$)
${}_0I^m_{Na}$	I^m_{Na} at $\psi^{mc} = 0$
E^m_{Na}	equivalent electromotive force for Na entry across the apical membrane or ψ^{mc} at which $I^m_{Na} = 0$ (mV)
$(Na)_m$	Na activity in the mucosal solution (mM)
$(Na)_c$	steady-state intracellular Na activity of the Na transporting cells (mM)
P^m_{Na}	steady-state permeability of the amiloride-sensitive Na entry pathway across the apical membrane (cm sec^{-1})
${}_0G^m_{Na}$	conductance of the amiloride-sensitive apical Na entry pathway when $\psi^{mc} = 0$ (mS/cm)[2]

Materials and Methods

Segments of descending colon were obtained from white rabbits (2–3 kg), which were killed by intravenous injection of pentobarbital. The colon was opened along its mesenteric border and rinsed free of intestinal contents. The outer muscle layers were stripped off and the resulting "partial mucosal strip" preparations were mounted in Ussing-type chambers as described by Frizzell, Koch and Schultz (1976).

The standard electrolyte solution bathing the mucosal surface of the tissue contained (mM): 140 Na; 124 Cl; 21 HCO_3; 5.4 K; 2.4 HPO_4; 0.6 H_2PO_4; 1.2 Mg; and 1.2 Ca. Solutions with lower Na concentrations were prepared by isosmolar replacement of Na with choline[2]. The serosal solution contained (mM): 140, K; 25, Cl; 46.7, SO_4; 21, HCO_3; 2.4, HPO_4; 0.6, H_2PO_4; 1.2, Mg; 1.2, Ca; and 57.1, mannitol. Since the K activity coefficient of the bathing solution with 50 mM SO_4 is approximately 0.54 (Fuchs et al., 1977; Lewis, Wills & Eaton 1978), the K activity of the serosal solution was approximately 76 mM, which is identical to the intracellular K activity reported by Wills et al. (1979) and confirmed in this laboratory (M. Duffey and S.G. Schultz, *unpublished observations*) for rabbit descending colon. All electrolyte solutions also contained 10 mM glucose and were gassed with 95% O_2 and 5% CO_2, resulting in a pH of 7.4 at 37 °C.

The isolated epithelia were short circuited using an automatic voltage clamp which was controlled by a computer (LSI 11/03, Digital Equipment Corporation) and could be open circuited, voltage clamped, or subjected to a train of current pulses by commands from the console terminal. The pulse train consisted of alternating polarity current pulses sufficient to clamp ψ^{ms} over the range of 0 to ± 200 mV in steps of 20 mV (e.g., $\psi^{ms} = 0, +20, 0, -20, 0, +40, 0, \ldots, 0, +200, 0, -200$ mV). Each pulse had a duration of 100 msec and the interval between pulses was 400 msec. The transepithelial current (I^{ms}) and the corresponding ψ^{ms} during each pulse were recorded by a chart recorder. The values of I^{ms} and ψ^{ms} 20 msec after the upstroke of each pulse were relayed to the computer via an analog-to-digital converter and stored for later processing. A description of the computer-driven voltage clamp and pulse generator has been reported previously (Thompson et al., 1982*a*).

The experimental protocol was as follows: When the short-circuit current, I_{sc}, had stabilized (usually 30–40 min after mounting the tissue), a current-voltage ($I-V$) relation was obtained by generating a current-pulse train across the tissue. Immediately following storage of the data, 10^{-4} M amiloride was added to the mucosal solution. After the I_{sc} had reached a new steady state (2–3 min after addition of amiloride) a second $I-V$ relation was obtained. The difference between the values of I^{ms} at a given ψ^{ms} is taken to represent the Na current across the apical cell membrane (I^m_{Na}), at that ψ^{ms} (Thompson et al., 1982*a*).[3]

Usually eight tissues from a single animal were mounted in separate Ussing-type chambers (Frizzell et al., 1976) and $I-V$ relations were generated as described above in the presence of one of five different mucosal Na concentrations (140, 70, 35, 17.5, or 8.75 mM). In some instances, amiloride was washed

[2] The Na activity coefficient of the solution bathing the luminal surface of the tissue was taken to be 0.71 (Robinson & Stokes, 1959).

[3] Since the solutions bathing the two sides of the epithelium had different ionic compositions, which gives rise to small diffusion potentials, I_{sc} is not identical to net Na absorption under these conditions. Thus, ${}_0I^m_{Na}$ was determined from the difference between I_{sc} in the absence and presence of amiloride.

out and the effects of a second Na concentration were examined on the same segment.

The $I-V$ relations of the amiloride-sensitive Na entry pathway were evaluated by fitting the parameters P^m_{Na} and $(Na)_c$ of the Goldman-Hodgkin-Katz (GHK) constant field flux equation for a single permeant ion

$$I^m_{Na} = -\left[\frac{P^m_{Na}\mathcal{F}^2\psi^{mc}}{RT}\right]\cdot\left[\frac{(Na)_m-(Na)_c\exp(\mathcal{F}\psi^{mc}/RT)}{1-\exp(\mathcal{F}\psi^{mc}/RT)}\right] \quad (1)$$

to the experimental values using nonlinear regression analysis assuming that $\psi^{mc}\approx\psi^{ms}$ (Thompson et al., 1982*a*; Thomas et al., 1983). According to this mathematical procedure, initial parameter estimates are adjusted so that the sum of squares of the differences between the experimental and calculated values converges to a minimum. The adjustments of the parameter estimates were the partial derivatives of the constant field flux equation with respect to each parameter. The initial estimates of $(Na)_c$ were obtained from the "reversal potential" E^m_{Na} (ψ^{mc} at which $I^m_{Na}=0$) using the Nernst equation and those of P^m_{Na} from the values of I^m_{Na} when $\psi^{mc}=0$ (i.e., ${}_0I^m_{Na}$) using Fick's law.[1]

The nonlinear least-squares approximation was performed on a Hewlett-Packard 2647A graphics terminal. A copy of the graphics could be obtained using a Hewlett-Packard plotter. All arithmetic means are given ± SEM.

Results

Examples of $I-V$ relations of the amiloride-sensitive apical Na entry pathway in the presence of a high and a low mucosal Na activity are illustrated in Fig. 1. At extreme positive and negative values of ψ^{mc}, the experimental values departed systematically from the predictions of the constant field flux equation. Therefore, the nonlinear least-squares approximation of the parameters of the constant-field flux equation to the experimental values was performed only over the range of voltages where the data conformed closely to this equation as judged by eye. This range was usually −120 to +100 mV; the latter is well beyond the values of E^m_{Na} encountered in these studies. When the experimental data could not be satisfactorily fit by the constant field flux equation over this range, the data were rejected; this was the case in about 20 to 25% of all experiments. It is noteworthy that the best results were obtained when the time interval between the pulse trains in the absence and presence of amiloride was as brief as possible.[4]

Since current and voltage were sampled 20 msec after the beginning of each pulse and because bipolar pulses were employed, it can be reasonably assumed that the imposed voltage gradients did not cause significant changes in intracellular ionic composition. The resulting values of $(Na)_c$ may therefore be considered to represent valid estimates of the steady-state intracellular Na activity.

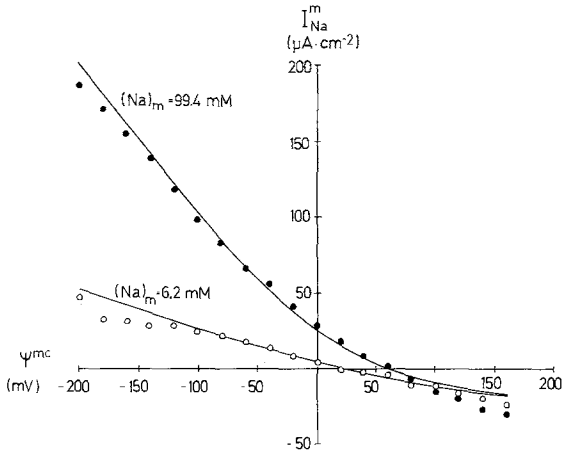

Fig. 1. Current-voltage relation of the apical Na entry pathway at $(Na)_m=99.4$ mM and 6.2 mM. I^m_{Na} is the difference in transepithelial current in the absence and presence of amiloride. The curves were calculated using an iterative curve-fitting procedure to Eq. (1)

From Fig. 1 it is clear that the reversal potential, E^m_{Na}, i.e., ψ^{mc} at which $I^m_{Na}=0$, and the slope conductance at $\psi^{mc}=0$ were considerably lower at the low mucosal Na activity than at the high mucosal Na activity.

Dependence of $(Na)_c$ and E^m_{Na} on $(Na)_m$

As discussed in Materials and Methods, inasmuch as the $I-V$ relation conforms to the GHK equation, $(Na)_c$ and P^m_{Na} can be derived by nonlinear regression analysis of the $I-V$ relations of the apical Na entry process. The results of all experiments are given in the Table.

When $(Na)_m=99.4$ mM, $(Na)_c$ averaged 11.9 ± 2.5 mM ($n=17$), in good agreement with the values reported earlier (Schultz, Frizzell & Nellans 1977; Thompson et al., 1982*a*). As $(Na)_m$ decreased, $(Na)_c$ fell in a curvilinear fashion (Fig. 2) which can be *empirically* described by the relation

$$(Na)_c=[(Na)_m\cdot(Na)_{c(m)}]/[K+(Na)_m] \quad (2)$$

where $(Na)_{c(m)}$ is the maximum $(Na)_c$ at "infinite" $(Na)_m$ and K is the value of $(Na)_m$ at which $(Na)_c$ is half-maximal. The solid curve illustrated in Fig. 2 is for the condition $(Na)_{c(m)}=15$ mM and $K=20$ mM. The significance of the dashed curve will be discussed below.

Since $(Na)_c$ and $(Na)_m$ are not linearly related, it follows that E^m_{Na} also changes with $(Na)_m$. As

[4] It is possible that under the conditions of these experiments, prolonged exposure to amiloride leads to changes in the conductance of pathways in parallel with the apical Na-entry pathway (Nage, Garci-Diaz & Essig, 1983). If so, the difference between I_{sc} in the absence and presence of amiloride would not represent ${}_0I^m_{Na}$.

Table. Effects of varying $(Na)_m$ on other parameters[a]

$(Na)_m$ (mM)	6.2	12.4	24.9	49.7	99.4
$(Na)_c$ (mM)	2.9±0.3	6.0±0.6	7.8±1.2	10.2±2.4	11.9±2.5
${}_0I^m_{Na}$ ($\mu A/cm^2$)	2.5±0.7	5.2±1.2	13.7±4.3	17.3±2.9	27.4±4.5
P^m_{Na} (10^{-6} cm/sec)	12.9±2.7	8.2±1.7	7.1±1.8	3.7±0.6	2.7±0.4
E^m_{Na} (mV)	22.9±3.5	22.1±3.7	34.7±4.4	49.0±7.0	65.4±5.6
n	14	14	12	9	17

[a] n represents the number of tissues studied.

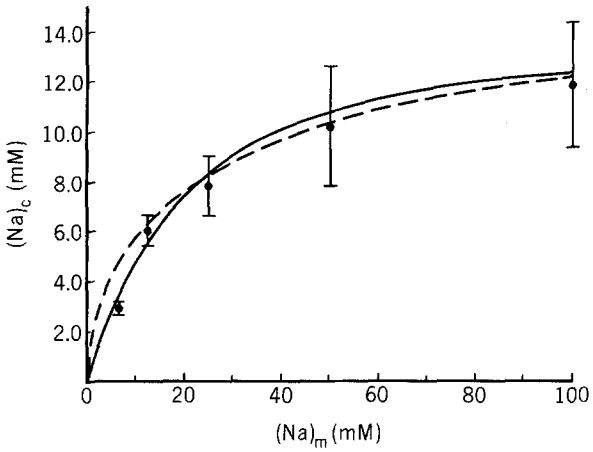

Fig. 2. Relations between $(Na)_m$ and $(Na)_c$. The solid curve was obtained using Eq. (2); the dashed curve corresponds to Eq. (8)

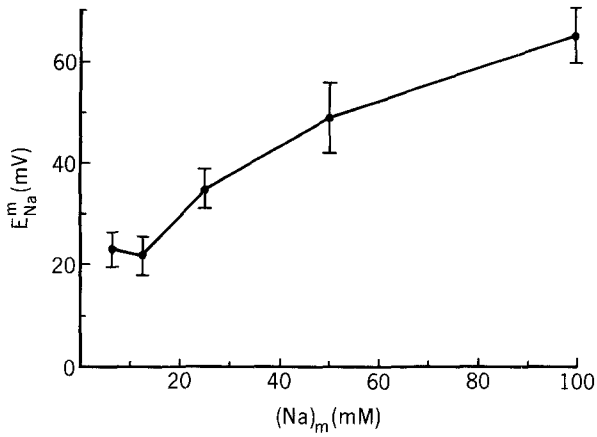

Fig. 3. Relation between $(Na)_m$ and E^m_{Na}

is apparent from Fig. 3, a proportional change in $(Na)_c$ was observed only at the two lowest values of $(Na)_m$ tested, whereas with a further increase in $(Na)_m$, E^m_{Na} also increased.

When $(Na)_m = 99.4$ mM, E^m_{Na} averaged 65 mV; Similar values were determined in the same epithelium by Schultz et al. (1977) and Wills et al. (1979) using microelectrodes.

P^m_{Na} as Functions of $(Na)_m$ and $(Na)_c$

When $(Na)_m = 99.4$ mM, P^m_{Na} averaged $2.68 \pm 0.40 \times 10^{-6}$ cm sec^{-1}. Using the same technique, P^m_{Na} was estimated to be 1.94×10^{-6} cm sec^{-1} in rabbit descending colon (Thompson et al., 1982*a*); 14.5×10^{-6} cm sec^{-1} when $(Na)_m = 28$ mM in frog skin (Fuchs et al., 1977); and, 5.2×10^{-6} cm sec^{-1} when $(Na)_m = 14$ mM in toad urinary bladder (Palmer et al., 1980). These values are in remarkably good agreement given the uncertainties of "true" apical membrane area.

A decrease in $(Na)_m$ was associated with a curvilinear increase in P^m_{Na} (Fig. 4*a*). This curvilinear relation between $(Na)_m$ and P^m_{Na} can be linearized by plotting $1/P^m_{Na}$ versus $(Na)_m$; therefore the relation between $(Na)_m$ and P^m_{Na} may be *empirically* described by the relation

$$P^m_{Na} = \left[\frac{P^m_{Na(m)} \cdot (Na)_{m(0.5)}}{(Na)_m + (Na)_{m(0.5)}} \right] \quad (3)$$

where $P^m_{Na(m)}$ is the maximum P^m_{Na} at $(Na)_m = 0$ and $(Na)_{m(0.5)}$ is the value of $(Na)_m$ at which P^m_{Na} is half-maximal. An analogous equation was employed by Fuchs et al. (1977) in their studies on frog skin. The maximum P^m_{Na} obtained in this manner was 13.3×10^{-6} cm sec^{-1}; $(Na)_{m(0.5)}$ was approximately 24 mM.

Thus, there is good agreement between the values of $(Na)_m$ at which the changes in $(Na)_c$ and P^m_{Na} are half-maximal. However, it should be emphasized that Eqs. (2) and (3) are empirically determined relations that, at present, are entirely descriptive and lack a formal, theoretical foundation. Thus, this agreement could be entirely fortuitous and without deeper meaning in terms of the complex, underlying mechanisms that regulate P^m_{Na} and $(Na)_c$.

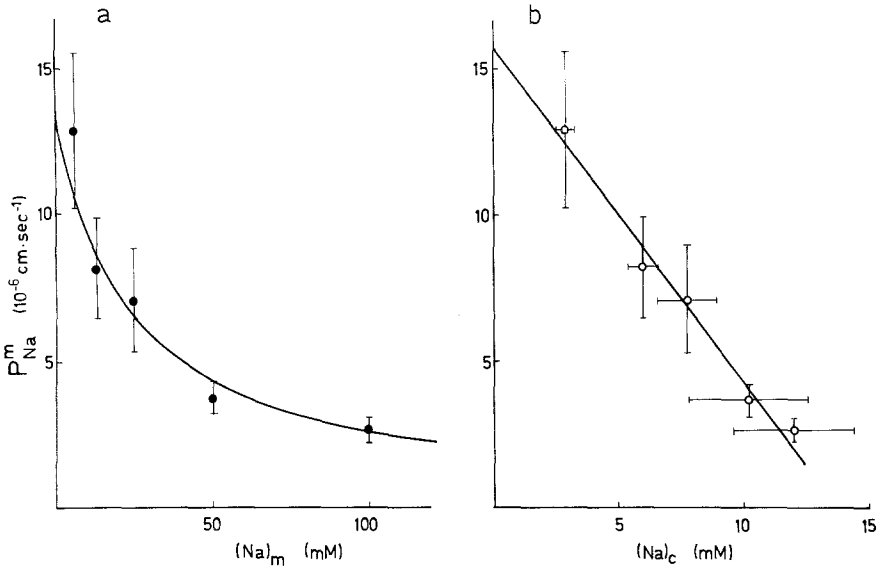

Fig. 4. Relation between P^m_{Na} and (*a*) $(Na)_m$ or (*b*) $(Na)_c$. The curve in *a* conforms to Eq. (3) and was obtained from a plot of $1/P^m_{Na}$ versus $(Na)_m$. The line in *b* was calculated by linear regression analysis

Figure 4*b* illustrates the relation between $(Na)_c$ and P^m_{Na}. Clearly, there is a negative linear correlation between these two parameters such that when $(Na)_c$ was high, P^m_{Na} was low and vice versa. The relation between $(Na)_c$ and P^m_{Na} conforms to the equation

$$P^m_{Na} = P^m_{Na(m)} - k(Na)_c \quad (4)$$

where, in this case, $P^m_{Na(m)}$ is the maximum P^m_{Na} at $(Na)_c = 0$, and k is a proportionality factor. The maximum P^m_{Na} calculated from this linear regression was 15.7×10^{-6} cm sec^{-1} and the value of $(Na)_c$ at which P^m_{Na} is half-maximal was approximately 7 mM. However, upon closer inspection, this relation between $(Na)_c$ and P^m_{Na} may be deceiving. Thus, when the individual values of P^m_{Na} and the corresponding values of $(Na)_c$ are plotted for each experiment (Fig. 5), we see that high values of $(Na)_c$ (>10 mM) were always associated with low values of P^m_{Na}, but that at low values of $(Na)_c$ (<9 mM), both high and low values of P^m_{Na} were observed. Clearly, because $(Na)_c$ increased with increasing $(Na)_m$, one cannot distinguish between a primary effect of $(Na)_m$ on P^m_{Na} ("self-inhibition" described by Fuchs et al., 1977), an effect of $(Na)_c$ on P^m_{Na} (the so-called "negative feedback") (*cf.* Schultz, 1981a), or some combination of these processes. This matter will be discussed further below.

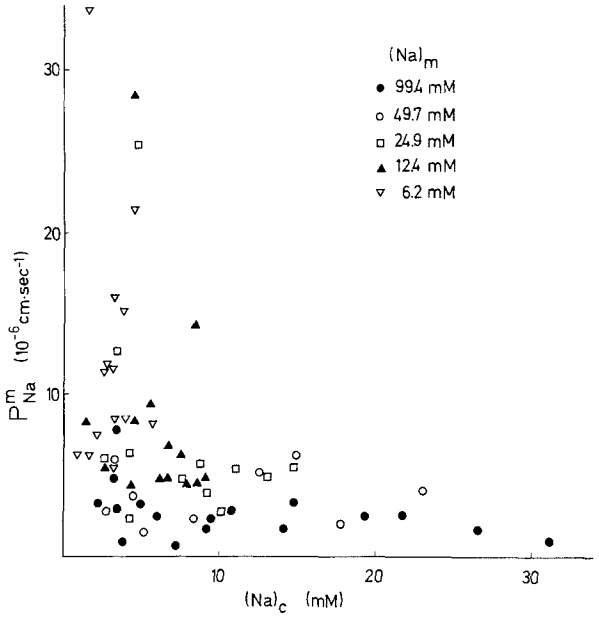

Fig. 5. Relation between P^m_{Na} and $(Na)_c$ of the individual experiments

Relation between P^m_{Na} and Apical Na Entry

When the apical membrane is short-circuited (i.e., $\psi^{mc} = 0$), Na entry may be described by

$$_0I^m_{Na} = \mathcal{F} P^m_{Na}[(Na)_m - (Na)_c]. \quad (5)$$

Thus, changes in $_0I^m_{Na}$ can result from changes in P^m_{Na} and/or changes in the difference in Na activities across the apical membrane.

The relation between spontaneous variations in $_0I^m_{Na}$, and P^m_{Na} when $(Na)_m = 99.4$ mM is illustrated in Fig. 6. Clearly, there is a linear positive correlation between $_0I^m_{Na}$ and P^m_{Na}, and the intercept does not differ significantly from the origin. The linearity of this relation implies that the spontaneous variations in rate of apical Na entry are the results of variations in apical Na permeability, and that the driving force for entry remains essentially constant. Indeed, there was no significant correlation

between ${}_0I^m_{Na}$ and $[(Na)_m-(Na)_c]$. Linear relations between spontaneous variations in ${}_0I^m_{Na}$ and P^m_{Na} were also found at the other mucosal Na activities tested. Thus, as reported previously (Thompson et al., 1982*a*), spontaneous variations in the rate of transcellular Na transport at fixed $(Na)_m$ are due entirely to parallel variations in P^m_{Na} or the chord conductance, ${}_0G^m_{Na}$; the thermodynamic driving force for Na entry under these conditions is constant.

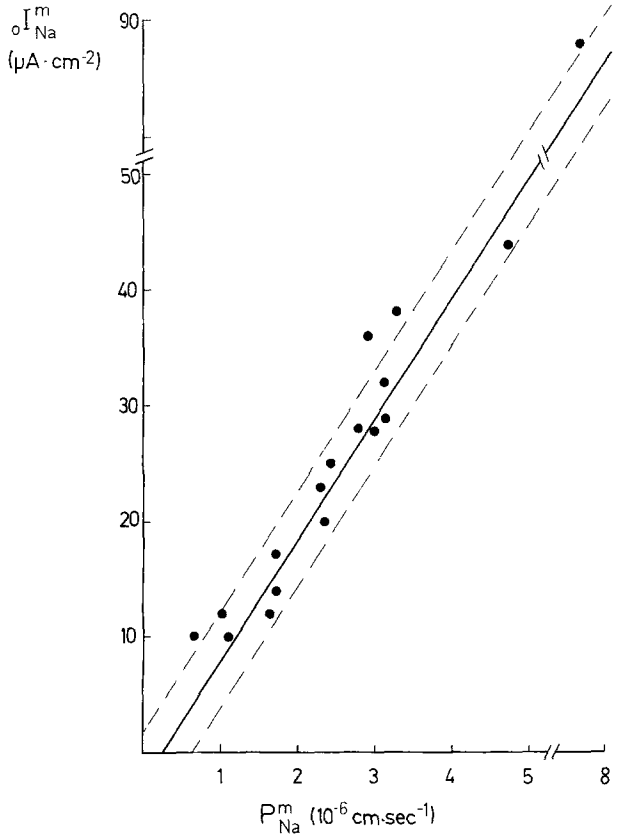

Fig. 6. Linear regression analysis of the relation between ${}_0I^m_{Na}$ and P^m_{Na} at $(Na)_m=99.4$ mM. The interrupted lines represent the standard deviations from the regression line. The intercept on the abscissa is not significantly different from the origin

Dependence of Transcellular Na Transport on $(Na)_m$ and $(Na)_c$

Under steady-state conditions, the Na currents across the apical and the basolateral cell membranes are assumed to be identical. Hence, we can establish not only the relation between apical Na entry and $(Na)_m$ but also the relation between basolateral Na exit and $(Na)_c$.

Figure 7*a* depicts the kinetics of the transapical Na current, ${}_0I^m_{Na}$, as a function of $(Na)_m$. Apical Na entry saturates as $(Na)_m$ increases and the relation conforms to the simple Michaelis-Menten relation

$${}_0I^m_{Na}=[{}_0I^m_{Na(m)}\cdot(Na)_m]/[K^m_{Na}+(Na)_m] \quad (6)$$

where ${}_0I^m_{Na(m)}$ is the maximum rate of Na entry across the apical membrane and K^m_{Na} is the value of $(Na)_m$ at which ${}_0I^m_{Na}=({}_0I^m_{Na(m)}/2)$. The curve illustrated in Fig. 7*a* corresponds to the values ${}_0I^m_{Na(m)}=50$ μA/cm² and $K^m_{Na}=80$ mM.

Under physiological conditions, when the basolateral membrane is *not* depolarized and ψ^{mc} is *not* zero, the maximum rate of Na entry is much higher (approximately 100 μA cm^{-2}) and K^m_{Na} is considerably lower (14 mM) (Frizzell & Turnheim, 1978). Hence, depolarizing the basolateral membrane by elevating the K concentration in the serosal bathing medium appears to markedly alter the kinetics of apical Na entry. This matter will be discussed further below.

The relation between basolateral Na exit, ${}_0I^s_{Na}$, and $(Na)_c$, on the other hand, does not conform to simple saturation kinetics; rather the curve is sharply sigmoidal (Fig. 7*b*). This relation suggests binding of Na to multiple sites on the transport

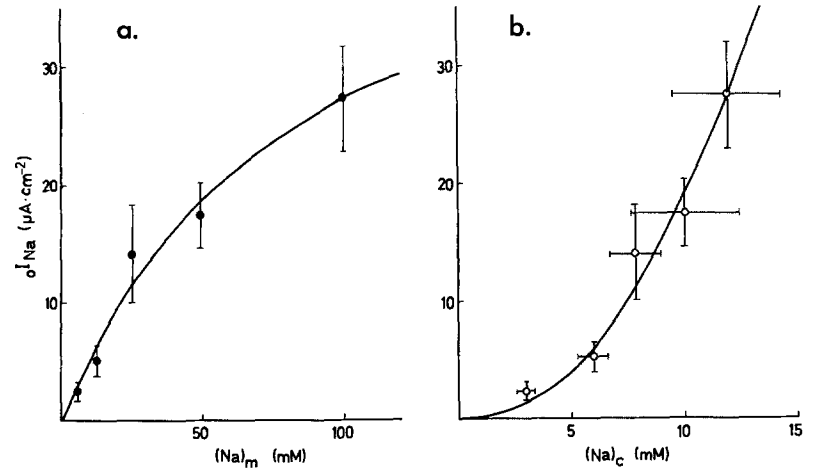

Fig. 7. Relation between transcellular Na transport (${}_0I_{Na}$) and (*a*) $(Na)_m$ *or* (*b*) $(Na)_c$. The curve in *a* was calculated by fitting the parameters of simple Michaelis-Menten kinetics to the data; the curve in *b* represents the best fit to Eq. (6)

mechanism. The stoichiometry of the interaction of Na with the basolateral Na extrusion mechanism can be calculated using the Hill equation, which is the simplified velocity equation for multisite enzymes, assuming strong cooperativity in substrate binding (Segel, 1975):

$$ {}_0I^s_{Na} = \left[\frac{{}_0I^s_{Na(m)}}{1+[K^s_{Na}/(Na)_c]^n} \right] \quad (7) $$

where n denotes the number of sites per transport unit, ${}_0I^s_{Na(m)}$ is the maximum rate of the Na exit process and K^s_{Na} is the value of $(Na)_c$ at which the rate of basolateral Na exit is half-maximal. From a Hill plot, which is simply a linearized version of Eq. (6) (Segel, 1975), n can be calculated, assuming a maximum Na pump rate of 170 μA cm^{-2}; this is the maximum rate of transcellular Na transport observed in this tissue under conditions when the basolateral Na extrusion mechanism is rate limiting for transcellular Na transport (Turnheim, Frizzell & Schultz, 1977; 1978; Frizzell & Turnheim, 1978; Frizzell & Schultz, 1978.[5]) The value of n derived in this manner was 2.4; K^s_{Na} was approximately 24 mM. Using these values and Eq. (6), the solid curve in Fig. 7*b* can be generated; this curve clearly provides an excellent fit to the data. Hence, under conditions of our experiments, 2–3 Na ions appear to interact with the basolateral Na extrusion mechanism.

Finally, because under steady-state conditions ${}_0I^m_{Na} = {}_0I^s_{Na} = {}_0I_{Na}$ Eqs. (6) and (7) can be combined to yield the equation

$$ (Na)_c = \frac{[{}_0I^m_{Na(m)}/({}_0I^s_{Na(m)} - {}_0I^m_{Na(m)})]^{1/n} K^s_{Na}}{\left[1+\left(\frac{{}_0I^s_{Na(m)}\, K^m_{Na}/(Na)_m}{{}_0I^s_{Na(m)} - {}_0I^m_{Na(m)}}\right)\right]^{1/n}}. \quad (8) $$

Inserting the values for ${}_0I^m_{Na(m)}$, K^m_{Na}, ${}_0I^s_{Na(m)}$, K^s_{Na} and n, given above into Eq. (8), we obtain the relation between $(Na)_m$ and $(Na)_c$ given by the dashed curve in Fig. 2, which clearly provides an excellent fit to the experimental data.

The importance of this observation cannot be over-emphasized! Equations (6) and (7) are simply empirical descriptions of the relations between the rates of Na transport and Na concentrations; the use of such descriptions, analogous to those employed in enzyme-kinetics, is a well established practice. The finding that two entirely independent, empirical and descriptive relations (i.e., the relation between $(Na)_m$ and ${}_0I^m_{Na}$, which is determined directly, and the relation between the *calculated* values of $(Na)_c$ and ${}_0I^s_{Na}$, which assumes that the maximum pump rate observed in "normal tissues" is not significantly affected in the presence of a high-K serosal solution) are consistent with the relation between $(Na)_c$ and $(Na)_m$ (Fig. 2) provides strong support for the internal consistency of the experimental data and the assumptions underlying their analysis.

Discussion and Conclusions

In the present study, both P^m_{Na} and $(Na)_c$ were derived in the presence of varying $(Na)_m$ from the $I-V$ relations of apical Na entry, which may be accurately described by the Goldman-Hodgkin-Katz constant field flux equation over a wide range of transapical electrical potential differences. In order to obtain the $I-V$ relations of the apical membrane, the technique originally introduced by Fuchs et al. (1977) was employed. In essence, a high serosal K concentration was used to depolarize the basolateral membrane and markedly reduce its electrical resistance. Consequently, the transcellular pathway may be considered to consist of only a single resistor, the apical cell membrane.[6] The justification for this approach has been extensively discussed (Fuchs et al., 1977; Palmer et al., 1980; Thompson et al., 1982*a*). Depolarization of the basolateral membrane by elevation of serosal K has been demonstrated in amphibian and mammalian urinary bladder (Higgins & Frömter, 1974; Lewis et al., 1978); concomitant with the depolarization, the electrical resistance of the basolateral membrane of toad skin and urinary bladder is reduced to unmeasurable values (Rawlins, Mateu, Fragachan & Whittenburg, 1970; Palmer et al., 1980).

Direct experimental support for this approach was provided for rabbit descending colon by use

[5] In the cited studies, the Na permeability of the apical membrane was "maximized" by the addition of amphotericin B to the mucosal solution, the replacement of Cl in the mucosal solution with a number of stimulatory anions such as SO_4, or treatment of the tissue with aldosterone, *in vitro*. Under each of these stimulatory conditions, the rate of active Na absorption averaged 170 μA cm^{-2} and could not be further increased by combining these stimulatory agents. Thus we conclude that this is the maximum pump rate under "normal, *in vitro*" conditions and only assume that this maximum is not affected by the high-K serosal solution employed in the present studies. As discussed in the text, the validity of this "ad hoc" assumption is supported by the internal consistency of the resulting data.

[6] As discussed by Thompson et al. (1982*a*, *b*), the apical membrane of rabbit colonic Na-absorbing cells possesses a significant ionic conductance in parallel with the amiloride-sensitive Na entry pathway. This complicates the analysis of $I-V$ data on nondepolarized cells but should not influence the interpretation of the present data where the apical membrane "itself" is voltage-clamped.

of microelectrodes (Thompson et al., 1982*a*). In the presence of a high-K solution on the serosal side of the epithelium, the electrical potential difference across the basolateral membrane was essentially zero and the conductance of this membrane was increased so that $\psi^{mc} \approx \psi^{ms}$ at all values of transepithelial currents. There was excellent agreement between the $I-V$ relations of the apical Na entry step determined in the presence of a high serosal K using the present technique and an "intracellular" technique, where changes in ψ^{mc} during the voltage pulse train were monitored with an intracellular microelectrode. The value of $(Na)_c$ when $(Na)_m = 99.4$ mM measured with both techniques did not differ (Thompson et al., 1982*a*). The value of $(Na)_c$ when $(Na)_m = 99.4$ mM determined in the present study is also in excellent agreement with that determined previously on the "nondepolarized" preparation (Thompson et al., 1982*a*).

Sodium Entry Across the Apical Membrane

As shown in Fig. 2, an increase in $(Na)_m$ is associated with a hyperbolic increase in $(Na)_c$ consistent with a maximum $(Na)_c$ of approximately 15 mM and a half-maximum $(Na)_c$ when $(Na)_m =$ 20 mM. Similar relations have been reported for two "leaky" epithelia where Na entry appears to be coupled to the cotransport of Cl or counter transport of H; namely, *Necturus* proximal tubule (Spring & Giebisch, 1977; Kimura & Spring, 1979) and *Necturus* gallbladder (Garcia-Diaz & Armstrong, 1980). Hyperbolic relations between $(Na)_m$ and $(Na)_c$ have also been reported for toad urinary bladder, a "tight" epithelium with an amiloride-sensitive, conductive Na entry mechanism (channel) (Li et al., 1982; Palmer et al., 1982); these observations were made on preparations exposed to a high-K serosal solution. On the other hand, Thomas et al. (1983) have found that $(Na)_c$ is independent of $(Na)_m$ in short-circuited *Necturus* urinary bladder when the serosal solution consisted of a normal amphibian Ringer solution. Further, preliminary observations on *Necturus* urinary bladder exposed to a high-K serosal solution suggest that, under this condition, $(Na)_c$ increases with increasing $(Na)_m$. Thus, it will be important to determine in tight epithelia whether depolarizing the basolateral membrane somehow interferes with a regulatory mechanism that maintains $(Na)_c$ relatively constant in the face of changing $(Na)_m$.

As shown in Fig. 3, increasing $(Na)_m$ is also associated with an increase in E^m_{Na}, which, in this preparation where ${}_0\psi^{mc} \simeq 0$, is the sole driving force for the entry process. Further, at any fixed value of $(Na)_m$, E^m_{Na} is relatively constant so that spontaneous variations in ${}_0I^m_{Na}$ are attributable entirely to parallel changes in P^m_{Na} (Fig. 6). The finding that spontaneous variation in the rate of active Na absorption at fixed $(Na)_m$ is attributable entirely to spontaneous variations in the Na conductance of the apical membrane is consistent with data reported for "nondepolarized" rabbit colon (Thompson et al., 1982*a*).

Concomitant with an increase in $(Na)_m$ there is a hyperbolic decrease in P^m_{Na} which can be described by Eq. (2). Similar relations between P^m_{Na} and $(Na)_m$ have been observed for frog skin (Fuchs et al., 1977), and toad (Li et al., 1982, Palmer et al., 1982) and *Necturus* urinary bladder (Thomas et al., 1983). Thus, while the driving force for diffusional Na entry increases with increasing $(Na)_m$, the permeability of the apical membrane decreases. The overall effect is saturation of the rate of Na entry $({}_0I^m_{Na})$ with increasing $(Na)_m$. These findings and interpretations are entirely consistent with the findings of van Driessche and Lindemann (1979) on isolated frog skin exposed to a high-K inner solution. Employing noise analysis, these investigators found that increasing the Na concentration in the outer bathing solution over the range 6–60 mM resulted in a linear increase in the conductance of individual (single) Na channels but a hyperbolic decrease in the number of open or active channels; the final result is saturation of ${}_0I^m_{Na}$ with increasing $(Na)_m$ in spite of the fact that the single channel conductance and driving force for Na entry increase.

Finally, it is of interest to compare the kinetics of Na entry in this depolarized preparation with that observed in tissues whose serosal surface is bathed with a normal Ringer solution (nondepolarized). As shown in Fig. 7*a*, ${}_0I^m_{Na}$ saturates with increasing $(Na)_m$ with a maximum rate of entry of 50 $\mu A/cm^2$ and a K^m_{Na} of approximately 80 mM. In the nondepolarized tissue, the maximum rate of entry is approximately 100 $\mu A/cm^2$ and a half-maximum rate is observed in the presence of 10–20 mM Na (Turnheim, Frizzell & Schultz, 1978; Frizzell & Turnheim, 1978). There are at least two factors that contribute to these differences. First, in the nondepolarized tissue, the driving force for Na entry is given by $({}_0E^m_{Na} - {}_0\psi^{mc})$ and is thus greater than the driving force when (as in the present experiments), $\psi^{mc} = 0$. Thus, when $(Na)_m =$ 99.4 mM, the total driving force for ${}_0I^m_{Na}$ in the nondepolarized tissue is approximately 100 mV (Schultz et al., 1977; Wills et al., 1979; Thompson et al., 1982*a*) compared to the value of 65 mV observed in the present study (the difference being

${}_0\psi^{mc}$). Second, inasmuch as the entry process conforms to the GHK equation, the chord conductance of the apical membrane to Na, ${}_0G^m_{Na}$, is a strong function of ψ^{mc} (*see* Fig. 1) and increases as ψ^{mc} becomes more negative. Thus, ${}_0G^m_{Na}$ will be greater under physiological conditions where ${}_0\psi^{mc}<0$ than when ${}_0\psi^{mc}=0$. In the nondepolarized rabbit colon when $(Na)_m=99.4$ mM, ${}_0\psi^{mc}$ averaged -39 mV and ${}_0G^m_{Na}$ averaged 0.58 mS/cm^2 (Thompson et al., 1982*a*); the latter is significantly larger than the value of ${}_0G^m_{Na}$ of 0.42 mS/cm^2 determined in the present studies when $(Na)_m=99.4$ mM but ${}_0\psi^{mc}\simeq 0$. Using the values for $(Na)_c$ and P^m_{Na} when $(Na)_m=99.4$ mM given in the Table, it can readily be shown that the chord conductance, G^m_{Na}, predicted by the GHK equation when $\psi^{mc}=-40$ mV is 0.54 mS/cm^2 in excellent agreement with that determined on the nondepolarized preparation. Further, inasmuch as ${}_0\psi^{mc}$ hyperpolarizes (becomes more negative) as $(Na)_m$ decreases (Thomas et al., 1983; Narvarte & Finn, 1980; Nagel, 1977) the difference between ${}_0G^m_{Na}$ in the depolarized and nondepolarized tissues will be larger at low $(Na)_m$ than at high $(Na)_m$; this will tend to reduce the value of $(Na)_m$ at which entry is half-maximal (i.e., K^m_{Na}) in the nondepolarized tissue compared to one in which ${}_0\psi^{mc}$ is clamped at zero.

Thus, the differences between the maximum rate of Na entry and the value of $(Na)_m$ at which a half-maximal rate is observed in depolarized and nondepolarized rabbit colon can be attributed, at least in part, to differences in the overall driving force for Na entry and the dependence of ${}_0G^m_{Na}$ on ${}_0\psi^{mc}$. Indeed these results suggest that, at least when $(Na)_m\approx 100$ mM, the properties of the apical membrane of the depolarized and nondepolarized preparations are very similar.

Effect of $(Na)_m$ on P^m_{Na}

In frog skin, there is compelling evidence that increasing $(Na)_m$ at constant $(Na)_c$ results in a decrease in P^m_{Na} (Fuchs et al., 1977; van Driessche & Lindemann, 1979) (so-called "self-inhibition"). There is also compelling evidence for a variety of epithelia that an increase in $(Na)_c$ at constant $(Na)_m$ also reduces P^m_{Na} (so-called "negative feedback") (*cf.* Schultz, 1981*a*). In the present experiments, increasing $(Na)_m$ was associated with a hyperbolic decline in P^m_{Na} as found by Fuchs et al. (1977) for frog skin but inasmuch as $(Na)_c$ also increased hyperbolically, it is not possible to definitively implicate $(Na)_m$ and/or $(Na)_c$ in the observed effect on P^m_{Na}. The data given in Fig. 5 indicate that the lowest values of P^m_{Na} are observed when $(Na)_m$ *alone* or *both* $(Na)_m$ and $(Na)_c$ are high. Further, P^m_{Na} at high $(Na)_m$ seems to be relatively constant in spite of large spontaneous variations in $(Na)_c$; on the other hand, at low $(Na)_m$, $(Na)_c$ is relatively constant while P^m_{Na} shows considerable variation. Thus, these results seem to suggest that under the present conditions P^m_{Na} is predominantly influenced by $(Na)_m$.

It is of interest to briefly consider these results in the light of current views regarding the mechanism responsible for the "negative feedback" of $(Na)_c$ on P^m_{Na}. Evidence has been presented for the presence of a Na–Ca countertransport mechanism at the basolateral membranes of toad (Chase & Al-Awqati, 1983; Arruda et al., 1982) and turtle urinary bladder (Arruda, Sabatini & Westenfelder, 1982), rat small intestine (Hildmann, Schmidt & Murer, 1982), *Necturus* renal proximal tubule (Lee, Taylor & Windhager, 1980), and frog skin (Grinstein & Erlij, 1978) whereby the movement of Na into the cell across the basolateral membrane energizes the uphill extrusion of Ca from the cell across that barrier. If, as in other tissues (Blaustein, 1974), this mechanism is rheogenic involving the counterflow of more than 2 Na per Ca, the driving force for Ca extrusion is derived from both the Na-chemical gradient and the electrical potential difference across that membrane. There is reasonable evidence that removal of Na from the serosal solution results in an increase in cell Ca and that this may be *directly* responsible for the decrease in P^m_{Na} observed under these conditions (Grinstein & Erlii, 1978; Lee et al., 1980; Taylor, 1981; Arruda et al., 1982). Thus, it is argued that an increase in $(Na)_c$, which results in a decrease in the electrochemical potential difference for Na across the basolateral membrane, could lead to an increase in cell Ca and that this may be the direct mediator of the "negative feedback" on P^m_{Na}. Lorenzen, Lee and Windhager (1981) have shown that an increase in $(Na)_c$ brought about by treatment of *Necturus* proximal tubule with ouabain is associated with an increase in cell Ca activity and Chase and Al-Awqati (1983) have recently demonstrated that Ca in the micromolar range inhibits Na entry through the amiloride-sensitive channels of apical membrane vesicles of toad urinary bladder; these findings lend strong support to the plausibility of the notion that cell Ca may be the direct mediator of the "negative feedback" phenomenon.

Currently, there is no direct evidence that an increase in $(Na)_c$ over the physiological range is associated with an increase in cell Ca. But, if this purported explanation for the "negative feedback" is correct, this mechanism would not be ex-

pected to play a major role in a preparation bathed with a nominally Na-free, high-K serosal solution and in which the basolateral membrane is depolarized. In this respect it is of interest that Palmer et al. (1980) reported that exposure of a depolarized preparation of toad urinary bladder to ouabain resulted in a sixfold increase in $(Na)_c$ but only a 20% decrease in P_{Na}^m. Thus, it may be that in these preparations the mechanism purportedly responsible for the "negative feedback" between $(Na)_c$ and P_{Na}^m is aborted.

Relation Between $(Na)_c$ and "Pump Activity"

It is generally accepted that the Na−K exchange pump in symmetrical cells is rheogenic and extrudes approximately 3 Na in exchange for 2 K for every ATP consumed (Thomas, 1972; Glynn & Karlish, 1975; Hoffman, Kaplan & Callahan, 1979).

Suggestive evidence that 3 Na ions are extruded from epithelial cells for every ATP consumed derives from numerous studies on the relation between the rates of active Na absorption and O_2 consumption. The findings that approximately 18 Na ions are actively transported per O_2 consumed is consistent with the stoichiometry of 3 Na ions per ATP (*cf.* Martin & Diamond, 1966; Macknight, DiBona & Leaf, 1980).

The relation between $(Na)_c$ and the rate of active Na extrusion by the basolateral pump mechanism, illustrated in Fig. 7*b*, is consistent with the notion that 2–3 Na ions interact with the pump per cycle. The value of $(Na)_c$ at which the pump rate is half-maximal (K_{Na}^s) is estimated to be 24 mM; this value is in good agreement with the value of 20 mM in human erythrocytes (Hoffman, Kennedy & Lunn, 1981), and Jørgensen (1980) has reported that the half-saturation constant of the cytoplasmic Na site of purified renal (Na−K) ATPase is approximately 26 mM. Similar sigmoidal kinetics between $(Na)_c$ and pump activity have been reported for frog skin (Nielsen, 1982) and for rabbit urinary bladder by Lewis and Wills (1981) and Eaton et al. (Eaton, 1981; Eaton, Frace & Silverthorn, 1982) employing different approaches; in these studies, the number of Na ions interacting with the pump per cycle was estimated to be 2.6–3.2 and K_{Na}^s ranged between 2–19 mM. Evidence for a 3 Na:2 K *transport* stoichiometry has also been presented for frog skin (Nielsen, 1979), rabbit urinary bladder (Lewis & Wills, 1981) and turtle colon (Kirk, Halm & Pauson, 1980). Further, Schultz (1981*b*) has presented an indirect argument for a 3 Na:2 K coupling ratio in nondepolarized rabbit colon.

In short, the results obtained on a variety of epithelia using widely different approaches strongly suggest that the stoichiometry of the basolateral Na−K pump is independent of pump rate and closely resembles that reported for nonepithelial cells. The sigmoidal relation between $(Na)_c$ and pump activity with a Hill coefficient of 2–3 clearly offers the advantage of permitting large changes in I_{sc} in the face of relatively small changes in $(Na)_c$.

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