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Na^+ Transport by Rabbit Urinary Bladder, a Tight Epithelium

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Summary. By in vitro experiments on rabbit bladder, we reassessed the traditional view that mammalian urinary bladder lacks ion transport mechanisms. Since the ratio of actual-to-nominal membrane area in folded epithelia is variable and hard to estimate, we normalized membrane properties to apical membrane capacitance rather than to nominal area (probably $1\ \mu F \sim 1\ cm^2$ actual area). A new mounting technique that virtually eliminates edge damage yielded resistances up to $78{,}000\ \Omega\,\mu F$ for rabbit bladder, and resistances for amphibian skin and bladder much higher than those usually reported. This technique made it possible to observe a transport-related conductance pathway, and a close correlation between trans-epithelial conductance (G) and short-circuit current (I_{sc}) in these tight epithelia. G and I_{sc} were increased by mucosal (Na^+) [$I_{sc} \sim 0$ when (Na^+) ~ 0], aldosterone, serosal (HCO_3^-) and high mucosal (H^+); were decreased by amiloride, mucosal (Ca^{++}), ouabain, metabolic inhibitors and serosal (H^+); and were unaffected by (Cl^-) and little affected by antidiuretic hormone (ADH). Physiological variation in the rabbits' dietary Na^+ intake caused variations in bladder G and I_{sc} similar to those caused by the expected *in vivo* changes in aldosterone levels. The relation between G and I_{sc} was the same whether defined by diet changes, natural variation among individual rabbits, or most of the above agents. A method was developed for separately resolving conductances of junctions, basolateral cell membrane, and apical cell membrane from this $G - I_{sc}$ relation. Net Na^+ flux equalled I_{sc}. Net Cl^- flux was zero on short circuit and equalled only 25% of net Na^+ flux in open circuit. Bladder membrane fragments contained a $Na^+ - K^+$-activated, ouabain-inhibited ATPase. The physiological significance of Na^+ absorption against steep gradients in rabbit bladder may be to maintain kidney-generated ion gradients during bladder storage of urine, especially when the animal is Na^+-depleted.

The study described in this and the following paper (Lewis, Eaton & Diamond, 1976) had three goals: to determine whether mammalian urinary bladder possesses ion pumps of physiological significance; to develop improved methods for studying electrical properties of epithelia; and to understand better the Na^+ transport mechanism that is apparently shared among many so-called "tight" epithelia.

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In amphibian urinary bladder, numerous studies, beginning with those of Leaf (1955), have demonstrated the physiological significance of ion pumps in the animal's salt and water economy. In contrast, mammalian urinary bladder is generally assumed to be an inert sac that lacks ion pumps, simply stores urine, and plays no active role in urine formation, although actual evidence relevant to this assumption is scanty and conflicting. If this assumption were correct, ion concentration gradients established by the kidney would tend to dissipate by passive diffusion between blood and urine during storage in the bladder. For example, in the experiments of Rapoport, Nicholson and Yendt (1960) a 3 mM NaCl solution introduced into the bladder of an anesthetized dog had increased in concentration to 8 mM NaCl after 3 hr, yet (Na^+) in dog urine is less than 3 mM under some physiological conditions. This dilemma could be resolved if there were an ion pump responsive to physiological conditions. Evidence for such a pump was provided by *in vitro* experiments of Wickham (1964) and Borzelleca (1965), who observed a spontaneous electrical potential difference and a net sodium flux under short-circuit conditions, and who also observed that aldosterone increased and metabolic poisons decreased this potential or flux. However, *in vivo* experiments performed by numerous workers have reported ion fluxes to be only down electrochemical gradients (Englund, 1956; Hlad, Nelson & Holmes, 1956; Rapoport *et al.*, 1960; Rosenfeld, Aboulafia & Schwartz, 1963; Hakim, Lifson & Creevy, 1965; Fellows & Turnbull, 1971; Fellows & Marshall, 1972; Turnbull & Fellows, 1972). Our first goal was to resolve this conflict, and to understand the physiological significance of ion transport in mammalian bladder.

Our second goal was to solve two methodological problems in studying epithelial ion transport: the problems presented by variable membrane folding and by edge damage. We shall show that normalizing membrane electrical parameters to tissue capacitance (as a correlate of effective membrane area) reduces variation introduced by variable folding. A method will be presented for virtual elimination of edge damage.

Towards the third goal of understanding the Na^+ pump that the mammalian bladder shares with other "tight epithelia" (Frömter & Diamond, 1972) such as frog skin, renal distal tubule and amphibian bladder, we have used transepithelial and intracellular electrical techniques. These techniques clarify the Na^+-specific entry pathway of the apical cell membrane. Some of these results have been reported in abstract or preliminary form (Lewis & Diamond, 1974, 1975; Lewis, Eaton & Diamond, 1975).

Materials and Methods

Dissection

Bladders of adult male New Zealand white rabbits weighing 2–3 kg were used in all experiments. Immediately after sacrifice of the animal by intravenous administration of sodium pentobarbital a 10-ml urine sample was drawn from the bladder for analysis, and the bladder was excised and washed three times (50 ml/wash) with warm $NaCl-NaHCO_3$ Ringer's solution (*see* Solutions). The bladder was then stretched on a rack immersed in this solution at 37 °C, and forceps and a razor blade were used under a binocular microscope to dissect away the thick muscle layers. Light microscopic examination of the resulting

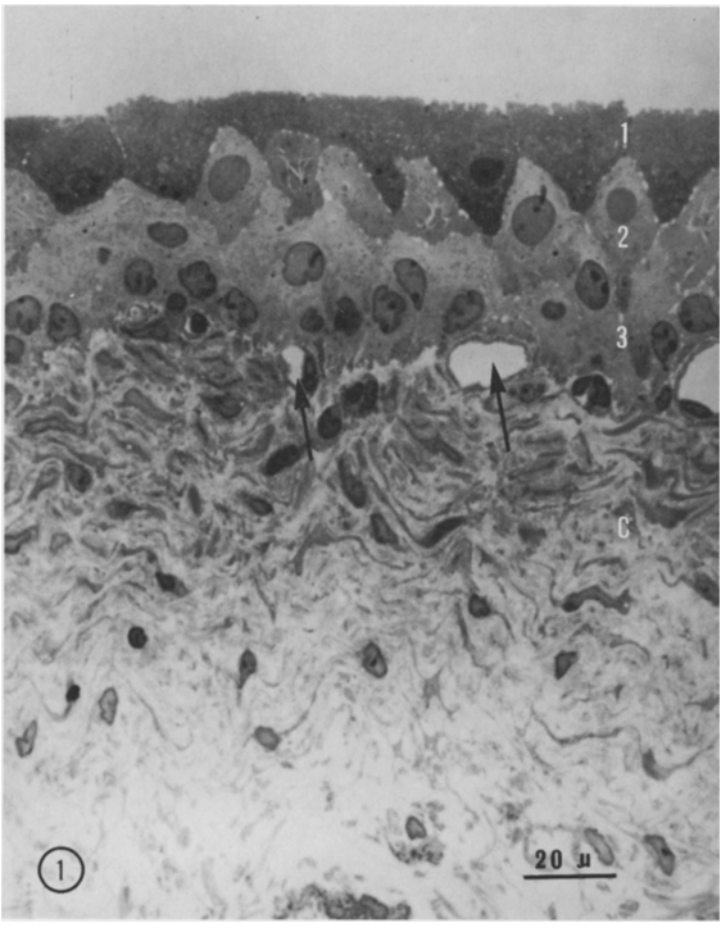


Plate 1. Light-micrograph of rabbit urinary bladder bathed in $NaCl-NaHCO_3$ Ringer's solution and then fixed in OsO_4. The three epithelial cell layers, which are easily distinguished by their varying intensity of staining are numbered beginning at the bladder lumen, where 1, 2 and 3 are the apical, middle and basal layers, respectively. The epithelial layers are seen to rest on such connective tissue (C) as remains after the muscle layers were stripped (*see* Materials and Methods). The arrows show part of the capillary bed. Magnification, 500 ×

stripped preparation after fixation showed that all three muscle layers had been completely removed. The remaining connective tissue was less than 80 μ thick. The epithelium (*see* Plate 1), consisting of three cell layers (Richter & Moize, 1963) whose relative significance is discussed in the following paper (Lewis *et al.*, 1976), was about 60 or 15 μ thick, depending on whether the bladder was unstretched or stretched, respectively. In unstripped bladders spontaneous muscle contraction caused fluctuations in transbladder potential and resistance. Stripping of the muscles abolished these fluctuations as well as eliminating responses of transbladder electrical parameters to application of the muscle contractant acetylcholine.

The experimental solution bathing the surface of the bladder that ordinarily faces the urine *in vivo* will be referred to as the mucosal solution while the solution bathing the surface normally exposed to the blood supply will be referred to as the serosal solution. Similarly, the epithelial cell membranes facing the urine or blood side will be cited as the apical or basolateral membrane, respectively.

Chambers and Mounting

The chambers (Fig. 1) were modified Ussing chambers of Plexiglas, maintained at 37 °C by circulating water jackets and resting in a groove in a Plexiglas platform. The volume of each chamber was 18 ml, and the exposed tissue area 2 cm^2. Solutions were stirred by Teflon-coated magnetic spin bars which sat in the flattened bottom of each chamber and were driven by external magnetic fleas linked to a motor. To mount the membrane, silicone grease (Dow Corning high-vacuum grease) was spread on the inside face of each of two plastic rings, one

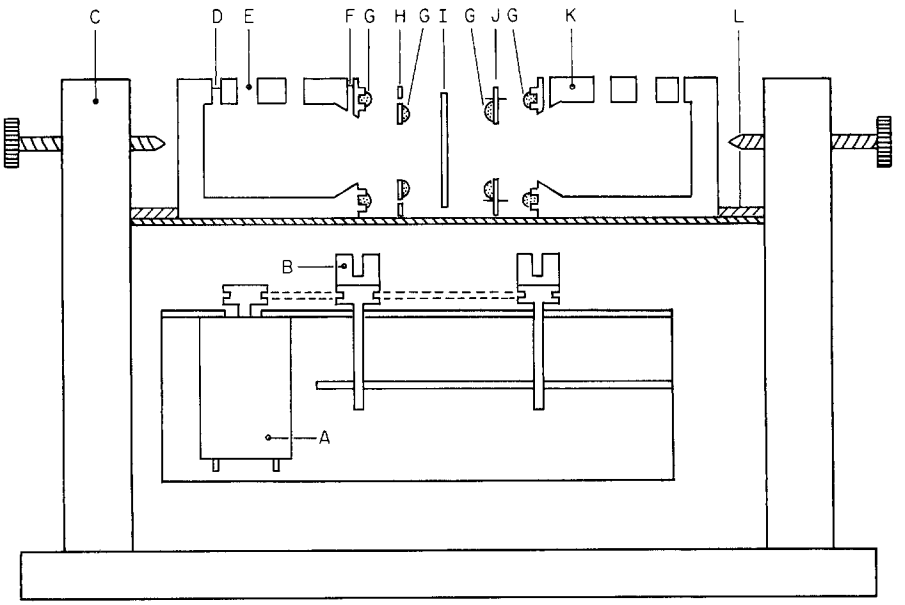


Fig. 1. Schematic cross-section of chambers. *A* = motor to drive external magnet *B* (internal magnetic spin bars not shown). *C* = vise. *D*, *E*, *F* = ports for current electrodes (*D*), changing solutions and gassing (*E*), and voltage electrodes (*F*). *G* = silicone sealant layer. *H*, *J* = plastic rings with 20 holes (*H*) or pins (*J*) around circumference. *I* = piece of bladder. *K* = chamber (water jacket is omitted for clarity). *L* = grooved platform into which ridge on bottom of chamber inserts to prevent lateral movement of chambers

with 20 pins and the other with 20 matching holes (*H and J* in Fig. 1). A piece of bladder was placed on one ring, and the other ring was placed on the opposite side of the bladder. After the ring- membrane-ring sandwich had been placed over the orifice of one chamber, the other chamber was brought up to the sandwich, and just enough pressure was applied to form a good seal between the rings and chambers, again using silicone sealant. The two chambers were located immediately over the magnetic stirrers and held in place by a vise. Solutions were introduced simultaneously into both chambers by open ports on the top (*E* in Fig. 1), through which gas was also continuously bubbled into the solutions. Membrane resistance and voltage were low immediately after mounting but rose to achieve a steady-state value by the end of two hours. This period represents the time required to achieve a seal between the mounting rings and the edge of the tissue.

Solutions

All solutions contained 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 11.1 mM glucose. The remaining solutes and their concentrations (in mM) were: "NaCl Ringer's solution", 132.5 NaCl, 7 KCl, 0.375 NaH_2PO_4, 2.125 Na_2HPO_4; "NaCl – $NaHCO_3$ Ringer's solution", 110 NaCl, 25 $NaHCO_3$, 7 KCl, 1.2 NaH_2PO_4; "choline Ringers's solution", 111.2 choline Cl, 25 choline HCO_3, 5.8 KCl, 1.2 KH_2PO_4; "methyl sulfate Ringer's solution", 110 Na methyl sulfate, 25 $NaHCO_3$, 3.5 K_2SO_4, 1.2 NaH_2PO_4, 4 mannitol. "NaCl – $NaHCO_3$ Ringer's solution" may be assumed to have been the one used in experiments where solution composition is not explicitly mentioned. "NaCl Ringer's solution" was gassed with 100% O_2, the other three solutions with 5% CO_2 – 95% O_2. All four solutions were buffered at pH 7.4 and were freshly prepared before each experiment. In experiments to vary pH without the complications introduced by varying (HCO_3^-), we replaced (HCO_3^-) with one of four other buffers: 4 mM Tris buffer at pH 8.2 and 7.4; phosphate at pH 7.4 (i.e., "NaCl Ringer's solution"); 10 mM MES (2-(N-morpholino)-ethanesulfonic acid) at pH 7.4 and 5.8; and addition of H_2SO_4 to unbuffered solutions below pH 5.8. Aldosterone (Calbiochem) was dissolved in 95% ethanol, of which microliter quantities were added to the bathing solution to achieve an aldosterone concentration of 19 μM. Control experiments showed that the same amount of ethanol without aldosterone had no effect on short-circuit current or transepithelial resistance. In retrospect, these concentrations of aldosterone are unphysiologically high, and the effect of aldosterone at physiological concentrations should be measured. Amiloride (Merck, Sharp and Dohme) was dissolved in NaCl Ringer's solution at 1 mM and added to the bathing solutions in microliter quantities (10^{-8} to 10^{-4} M final concentration). Back-to-back 50 ml syringes were used to change bathing solutions in the chambers without change of volume. If the solution changes were not made in this way, the resulting pressure difference across the bladder caused long transients or irreversible changes in transepithelial resistance.

Electrical Methods

Transepithelial potential differences (p.d.'s) were measured with a Keithley 610B electrometer connected to Ag – AgCl electrodes 5 mm from the tissue. Since in our experiments chloride activity gardients were always absent, and gradients of other ions were usually absent, no correction for differences in electrode potentials was needed. Current was measured with a Keithley 604 differential electrometer and was passed by Ag – AgCl electrodes on opposite sides of the membrane and at rear of the chamber. To measure short-circuit current (I_{sc}), a voltage-current clamp (Walker, Eisenman & Sandblom, 1968) was used to maintain the p.d. at zero. Since series resistance including solution between the voltage-sensing electrodes) was only 23–30 Ω but membrane resistance always exceeded

2500 Ω and was generally 4000–5000 Ω and often higher, no correction was made for a voltage drop in the adjacent solutions. The time courses of I_{sc} and of the spontaneous open-circuit p.d. (V_{sp}) were recorded on Varian and Keithley 370 paper chart recorders, respectively. The bladder was normally left on open circuit, and I_{sc} was measured at 15-min intervals. From about 9 hr after dissection the preparation began to deteriorate, as gauged by decrease in I_{sc} and R.

To measure transepithelial resistance R and time constant τ ($=RC$, where C is capacitance), a square current pulse of duration 1 sec and magnitude <8 μA was supplied by a Devices stimulator 2533 controlled by a Devices Digitimer. The current pulse and time-dependent voltage trace were displayed on a Tektronix D 15 storage oscilloscope for measurement. The voltage trace proved to be approximately linear on a semilogarithmic plot against time, justifying calculation of a single exponential time constant τ. R was calculated from the steady-state voltage and Ohm's law, while C was calculated as τ/R.

Nonlinearity of the current-voltage relation has been well documented in toad urinary bladder (Bindslev, Tormey, Pietras & Wright, 1974) and some other tight epithelia. This nonlinearity involves reversible, time-dependent resistance decreases when large voltage pulses (200–300 mV) are applied. Bindslev *et al.* (1974) concluded that these resistance changes involve both the tight junctions and the cell membranes. We found a similar time-dependent resistance decrease to occur in rabbit urinary bladder, but at much lower voltages. Transepithelial resistance at a series of holding voltages ranging from -100 to $+100$ mV was measured by 1 sec square current pulses adjusted to give a voltage response ΔV of

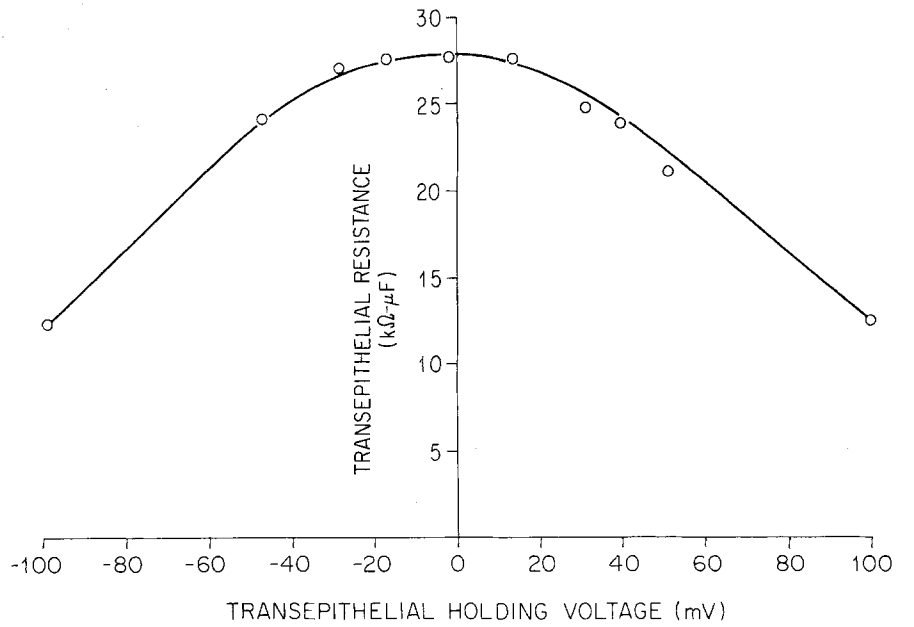


Fig. 2. Current-voltage relation in NaCl–$NaHCO_3$ Ringer's solution. A constant voltage of up to 100 mV was maintained across the bladder (abscissa; positive values mean serosa positive to mucosa). Square current pulses of 0.8 μA height, 1 sec duration, were applied on top of this holding voltage. The resistance (ordinate, in kΩμF; *see* p. 8 for discussion of why resistance is normalized to membrane capacitance rather than to area) was calculated from the voltage change after 1 sec. For holding voltages less than 20 mV the voltage change reached an asymptote. For holding voltages above 20 mV the voltage change declined with time after reaching a maximum, because of time-dependent and voltage-dependent resistance changes

± 4 mV (current $\leqq 1$ μA). Transepithelial R proved to be independent of holding voltage only up to holding voltages of about ± 20 mV. At higher holding voltages R declined with time, and the R value read off at 1 sec was voltage-dependent (Fig. 2). We do not know the anatomical location of these resistance changes, but we suspect the tight junctions (Lewis *et al.*, 1976). All conductance values reported in this paper were therefore measured at zero holding voltage (i.e., in the short-circuited condition) and with small (0.8 μA) current pulses as described in the provious paragraph. Clearly, conductance measurements on open circuit could not be meaningfully compared among different bladders, since the spontaneous voltage varies from 20 to 75 mV.

Analytical Methods

At the end of each experiment the exposed tissue was blotted twice against tissue paper, cut out, weighed wet, and then dried in an oven at 97 °C to constant dry weight.

Isolated membrane fragments were prepared for $Na^+ - K^+$-activated ATPase assay by scraping epithelial cells from the bladder, homogenizing the cells in chilled Tris buffer (20 mM Tris, pH 7.4), centrifuging (at $20{,}000 \times g$ for 16 min) and discarding the supernatant, resuspending, and again centrifuging (at $20{,}000 \times g$ for 16 min) and discarding the supernatant, resuspending, and again centrifuging (at $20{,}000 \times g$ for 16 min) and discarding the supernatant. The assay was by the method of Fujita, Matsui, Nagano and Nakao (1971).

Plasma samples were taken from rabbit's ear vein, allowed to coagulate for 30 min, and then centrifuged for 10 min. (Na^+) and (K^+) in urine and plasma samples were measured by flame photometry (instrumentation Laboratory model 143).

Throughout, statistical variation is expressed by standard errors of the mean.

Tracer Methods

Carrier-free ^{22}Na or else ^{36}Cl (100 μCi, New England Nuclear) were diluted with $NaCl - NaHCO_3$ Ringer's solution to make a 5 ml stock solution. To 17.5 ml of bathing solution in one chamber, termed the efflux chamber, was added 0.5 ml of stock solution at $\tau = 0$. Immediately, 0.5 ml and 20 μl samples were taken from the influx and efflux chambers, respectively, placed in a tared planchet, weighed, and dried. This procedure was repeated at the end of each experiment. Because fluxes across the bladder are very low, the counts in the efflux chamber at the beginning and end of each experiment were virtually the same. At 30-min intervals 0.5 ml samples were taken from the influx chamber (and replaced by 0.5 ml isotopically cold solution), placed in a tared planchet, weighed, and dried. Experiments lasted 5.5 hr, of which the first 2.5 hr were on open circuit, the remaining 3 hr on short circuit. The first 0.5 hr period on short circuit was not used for flux calculations. Samples were counted to 5% accuracy (because of very low fluxes and activities) in a Nuclear Chicago model 1042 planchet counter. Since the specific activity ratio between the influx and efflux chamber samples was *ca.* 0.2%, no correction was made for isotope back-flux. The Na^+ or Cl^- flux J (moles/sec) was calculated as

$$J = (N/S)(c_{t_2} D_{t_2} - c_{t_1} D_{t_1} K)/t_2 - t_1)$$

where N is the number of moles of Na^+ or Cl^- in the efflux solution, S the total number of counts/min of ^{22}Na or ^{36}Cl in the efflux solution, t_x the sample time, c_{t_x} counts/min of the 0.5 ml sample of influx solution at time t_x, D_{t_x} the ratio of the total influx solution volume to the sample size, and K a factor correcting for dilution by the replacement of the previous sample with cold solution. In six experiments the average coefficients of variation in flux and in I_{sc} were found to be approximately the same ($\pm 13\%$ and $\pm 15\%$, respectively), so that most variation in fluxes may reflect variation in I_{sc}.

Results

In this section we first discuss the solution of two methodological problems: correction for variable folding, by normalizing membrane parameters to capacitance; and elimination of edge damage. We next present six types of experiments designed to identify the actively transported ions. Finally, three physiologically significant variables [dietary Na^+ intake, urine pH and urine (Ca^{2+})] are shown to affect I_{sc} and R according to the same reciprocal relation as defined by the effects of other agents, suggesting a transport-related conductance pathway in the apical cell membrane.

Advantages of Using Capacitance to Normalize Membrane Electrical Parameters

Often, membrane electrical parameters such as I_{sc} or conductance are expressed per unit area of membrane. In epithelia this procedure has two disadvantages. First, an epithelium is not perfectly flat but is folded, so that effective area is greater than nominal area of exposed tissue. Second, it is impossible to avoid some variation in stretch and hence in folding between preparations, and this introduces scatter into parameter values related to nominal area. Alternative normalization procedures are to relate parameters to tissue dry weight or wet weight, or to attempt to mount preparations at constant stretch. In this section we show that normalization to membrane capacitance offers advantages.

If membranes of the same thickness and dielectric constant are compared, capacitance C is proportional to membrane area. Hence C, which can be measured as τ/R, recommends itself as a measure of effective area. In principle, however, this measure could present complications in epithelia. The simplest plausible equivalent circuit of an epithelium consists not just of a single resistor and single capacitor, but of two series resistor-and-capacitor elements (the apical and basolateral cell membranes) in parallel with a shunt resistor (the "tight junction"). This circuit is not equivalent to a single RC circuit. Its voltage time course following a square current pulse cannot be generally described by a single exponential but instead by a lengthy equation that we have derived from impedance analysis. If τ is nevertheless calculated as the time to achieve $100\,(1-1/e)=63\,\%$ of the steady-state voltage and the value of one circuit resistor is changed, the value of the effective circuit capacitance (calculated as $C=\tau/R$)

will also change, even though the value of neither individual capacitor in the circuit has changed (*see* Appendix for details). In addition, the meaning of effective area calculated in this way is ambiguous for an epithelium, since the actual area and capacitance of the basolateral cell membrane are generally several times those of the apical cell membrane.

Despite these difficulties in principle, the observed fact is that the voltage time course following a square current pulse in rabbit urinary bladder does approximately fit a single exponential. This is in part because junctional resistance is greater than 300,000 $\Omega\mu F$ or effectively infinite (Lewis *et al.*, 1976), so that the lengthy complete equation reduces to:

$$V_T(t) = V_b e^{-t/R_b C_b} + V_a e^{-t/R_a C_a}. \quad (1)$$

Here V_T, V_b and V_a are the time-dependent voltages across the whole epithelium, basolateral cell membrane, and apical cell membrane, respectively, while R and C are the resistance and capacitance of these cell membranes (subscripts b or a). In addition, we shall show (Lewis *et al.*, 1976) that when I_{sc} is very low (e.g., after addition of amiloride), R_a is much greater than R_b and Eq. (1) reduces to

$$V_T(t) \sim V_a e^{-t/R_a C_a}.$$

Under these circumstances $V_T(t)$ is expected to follow a single exponential, and the effective capacitance calculated from this exponential as τ/R (taking R as the transepithelial resistance) approximates the capacitance of the apical cell membrane. Therefore, we routinely measured C of each preparation in the presence of amiloride.

Fig. 3 illustrates the advantage of capacitance over dry weight for normalization. Both C and dry weight were measured for many preparations mounted at various degrees of stretch. For preparations under only slight stretch (dry weight >2.2 mg/cm^2), C increased approximately linearly with dry weight (Fig. 3), and we noted macroscopic folding of the bladder surface. In this range, evidently, variation in stretch is accommodated by macroscopic folding and unfolding. However, below 2.3 mg/cm^2 C changed much more slowly than dry weight: an increase in dry weight by 260% (from 0.7 to 2.3 mg/cm^2) was accompanied by only 14% increase in C (from 1.1 to 1.25 μF/cm^2). In this range macroscopic folds were absent, but the epithelial cells simply became flatter as they were stretched, so that change in dry weight was much greater than change in effective area or capacitance. (The number of cell layers was always three at any degree of stretch: *cf.* Richter & Moize, 1963.) Thus, normalization to dry weight would introduce systematic errors. In addition, when we

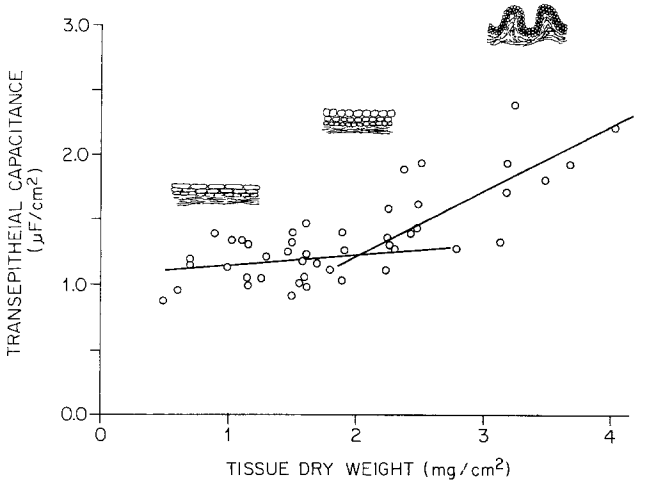


Fig. 3. Bladders were mounted at various degrees of stretch, and capacitance (ordinate) and tissue dry weight (abscissa) were measured and expressed relative to nominal tissue area (area of hole in chambers). Lower abscissa values correspond to more highly stretched tissues. The sketches show the appearance of the three-layered epithelium at various degrees of stretch. Note that the slope of the capacitance-*vs.*-dry-weight relation becomes steeper at low stretch values where tissue folding appears. *See text* for discussion

attempted to mount bladders at constant stretch, the coefficient of variation was larger for dry weight ($\pm 40\%$) than for capacitance ($\pm 24\%$).

In the remainder of these papers we report membrane resistance and I_{sc} as values normalized to membrane capacitance measured after addition of amiloride (in units $\Omega\,\mu F$ and $\mu A/\mu F$, respectively). As discussed, this capacitance measurement corresponds approximately to the capacitance of the apical membrane. The average expected capacitance value of *ca.* 1 $\mu F/cm^2$ for biological membranes (Fettiplace, Andrews & Haydon, 1971) may be used to estimate the corresponding R and I_{sc} values related to actual apical membrane area. The average measured capacitance for rabbit bladder related to exposed chamber area, 1.36 ± 0.05 ($n = 44$, SEM) $\mu F/cm^2$ is higher than 1 $\mu F/cm^2$ because of folding of the actual membrane.

Edge Damage

Any damage to the edge of the tissue, where it is compressed or crushed between the chambers, will be associated with a conductance. The error introduced by this edge-damage conductance into measurements of epithelial conductance will be proportionately greater, the lower the conductance of the native epithelium. The edge damage problem is therefore

severe in so-called tight epithelia with low native conductances (Dobson & Kidder, 1968; Walser, 1970). Helman and Miller (1971) found a skin adhesive effective in eliminating edge damage in frog skin, a relatively nondistensible epithelium. For rabbit urinary bladder, however, the skin adhesive failed to hold the tissue to the mounting rings at high stretch, made it difficult to readjust the placement of the tissue, and was hard to clean off the mounting rings.

Initially, we attempted to mount bladders using vaseline as a sealant. This technique never yielded transepithelial resistances above 30,000 Ω μF, even after amiloride (actual range, 11,000–28,000 Ω μF, the value being correlated with I_{sc} as will be illustrated in Fig. 11 from Lewis *et al.*, 1976). When the bladder was mounted with silicone sealant and only gentle pressure, as described under Materials and Methods, and when the conductance pathway in the epithelial cell membranes linked to Na^+ transport was blocked by amiloride as discussed in later sections, bladder resistance was up to 78,000 Ω μF. This represents a minimal value for edge-damage resistance, and would be the actual value if junctional resistance and residual cell membrane resistance were infinite. Actually, virtually all of the conductance associated even with this high resistance proves to be in the cell membranes. The measured resistance associated with edge damage plus junctions will be shown to be of the order of 300,000 Ω μF (Lewis *et al.*, 1976), i.e., at the limit of being detectable.

Is it possible that these high resistances and the gradual increase in resistance during the first two hours after mounting, are not due to elimination of edge damage but to silicone spreading over most of the exposed membrane surface and thus greatly reducing the effective area of membrane? To distinguish these two possibilities, we compared total membrane conductance in the presence of amiloride ($g_{tA}=g_e+g_j+g_{mA}$) and in the absence of amiloride ($g_t=g_e+g_j+g_m$), where g_e represents edge-damage conductance, g_j junctional conductance, and g_{mA} or g_m cell membrane conductance with or without amiloride. The remainder of this and the following paper will demonstrate that amiloride acts only to decrease g_m and does not affect g_e or g_j: i.e., $\Delta g=g_t-g_{tA}=g_m-g_{mA}$. If the decrease in g_t with time after mounting were due to an effective decrease in exposed membrane area, then Δg_t would decrease with time. If, however, g_t decreased with time because of elimination of edge damage (g_e), then Δg_t would remain independent of time. In practice, we found that Δg_t remained constant with time after mounting, although g_t itself decreased 30-fold. Therefore, the increase in resistance with time after mounting is due entirely to sealing of edge damage and not to a reduction in effective

membrane area. This conclusion is corroborated by the facts that membrane capacitance, a measure of effective membrane area (p. 8) also remains independent of time after mounting; and that we always looked for silicone spread across the membrane, and never observed any.

We have found this same technique useful in eliminating edge damage in other tight epithelia, such as frog skin and frog urinary bladder. The resistance values we have obtained for these epithelia (Lewis, Clausen & Diamond, 1975: up to 70,000 Ω μF in frog bladder, and up to 50,000 Ω μF in frog skin even in chloride Ringer's solution) are considerably higher than the values usually reported in the literature, suggesting that a significant fraction of the conductance usually measured for these tissues is an artifact of edge damage. With edge damage eliminated, it has been possible to demonstrate clearly a Na^+-dependent, aldosterone-stimulated, amiloride-inhibited conductance pathway in the apical membrane that had previously been detected only with difficulty (Civan & Hoffman, 1971) in tight epithelia. Higgins, Cesaro, Gebler and Frömter (1975) independently developed the same technique for eliminating edge damage and similarly succeeded in observing this transport-linked conductance pathway.

Identification of Active Transport Systems

In NaCl-$NaHCO_3$ Ringer's solution the serosal solution was always electrically positive to the mucosal solution, but numerical values of electrical properties varied among bladders: spontaneous voltage V_{sp} from 20 to 75 mV, I_{sc} from 1.5 to 20.5 μA/μF, and transepithelial resistance from 7,000 to 40,000 Ω μF. The significance of this spontaneous variation will be discussed on p. 32. To identify the ion pumps responsible for this V_{sp} and I_{sc}, we studied six sets of phenomena: effects of ion substitutions, amiloride, hormones, and metabolic inhibitors on I_{sc}; $Na^+ - K^+$-activated ATPase activity; and tracer fluxes.

1. *Effects of ion substitution on* I_{sc}. (a) *Na^+*. Na^+ was partly or completely replaced mole-for-mole with choline, symmetrically in both bathing solutions, by mixing NaCl – $NaHCO_3$ and choline Ringer's solutions in various proportions. Each experimental period was preceded and followed by a control period with NaCl – $NaHCO_3$ Ringer's solution. Both the control and experimental periods lasted until I_{sc} had reached a new steady state; this took about 30 min. About 90% of the change in I_{sc} was complete within the time required to replace solutions (5 min), while the remaining 10% of the change was slower. Since the control I_{sc} varied

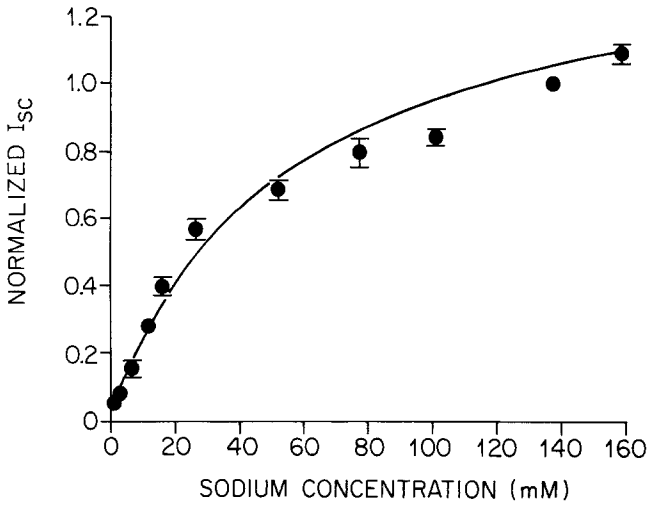


Fig. 4. Normalized I_{sc} as a function of (Na^+) was varied by symmetrical replacement with choline in both bathing solutions. Normalized I_{sc} means the ratio of I_{sc} at the test Na^+ concentration (abscissa) to I_{sc} in the same bladder at (Na^+)=136.2 mM. Vertical bars give the SEM ($n=6$). The solid line is the best fit of experimental points to Michaelis-Menten kinetics ($K_m=40$ mM, $V_{max}=1.35$). Evidently, the I_{sc} is almost completely dependent on Na^+

among preparations between 2 and 10 μA/μF, the experimental current I_{exp} was normalized to the average of the preceding (I_B) and subsequent (I_A) control currents, by calculating a normalized value $I_{exp}/[(I_B+I_A)/2)]$.

As shown in Fig. 4, normalized I_{sc} increased with increasing (Na^+) and achieved a half-maximal value at (Na^+)=40 mM. The best fit of the experimental points to Michaelis-Menten kinetics (solid curve in Fig. 4) is only fair, as is also true in frog bladder and skin. Inhibition of I_{sc} is 95% complete at zero (Na^+). The remaining 5% of I_{sc} might reflect either the presence of tissue Na^+ that has not yet been washed out, or else very slow transport of some ion other than Na^+. The effects of Na^+-choline substitutions were exerted entirely from the mucosal surface: serosal substitutions were ineffective. In two experiments partial symmetrical replacement of Na^+ by K^+ inhibited I_{sc} to the same degree as did corresponding replacement by choline, suggesting that K^+ is not actively pumped.

Normalized transepithelial conductance G_T also increased with increasing (Na^+) (Fig. 5). Comparison of the two curves in Fig. 5 illustrates that the change in G_T with (Na^+) is larger in bladders with higher spontaneous I_{sc} values. This hints at the existence of a Na^+ conductance pathway associated with Na^+ pumping, as will be documented further (Fig. 18, p. 30).

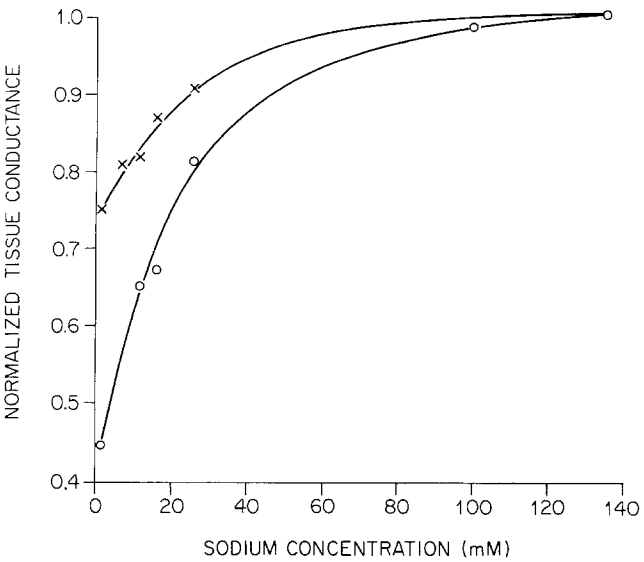


Fig. 5. Normalized conductance (ratio of G at test Na^+ concentration indicated on abscissa, to G in same bladder at $(Na^+)=136.2$ mM, as a function of (Na^+) (varied by symmetrical replacement with choline in both bathing solutions). The two curves refer to two different bladders, with $I_{sc}=5$ μA/μF (○) or 2.8 μA/μF (×) at $(Na^+)=136.2$ mM. Conductance changes are greater in the bladder with higher I_{sc}. However, the (Na^+) concentrations required for half-maximal reduction in G are approximately the same (22 and 18 mM for the low-I_{sc} and high-I_{sc} bladders, respectively)

(b) *Cl^-*. The anion $CH_3SO_4^-$ is relatively large, has a free-solution mobility only about 64% that of Cl^-, and might therefore be less permeant than Cl^-. If Cl^- were actively transported, replacement of Cl^- by $CH_3SO_4^-$ would probably increase or decrease I_{sc} depending on the direction of Cl^- transport, since $CH_3SO_4^-$ is not known to be subject to active transport in other tissues. If Cl^- moved passively but were sufficiently permeant to provide most of the counterions for Na^+ transport, replacement of Cl^- by a less permeant anion would increase V_{sp} and R. If Cl^- were not actively pumped and were relatively impermeant, replacement of Cl^- by $CH_3SO_4^-$ would have negligible effect on I_{sc}, V_{sp} and R. This last alternative proved to be the actual outcome: the respective changes in these parameters in three experiments were $+2\pm1$%, $+5\pm7$%, and $+1\pm2$% (average ± SEM), none of these changes being statistically significant.

(c) *HCO_3^-*. Symmetrical removal of HCO_3^- from both bathing solutions (replacing it with H_2PO_4 or MES) reversibly decreased I_{sc} and increased R (Fig. 6). This effect was mainly dependent on HCO_3^- in the serosal solution: removal of serosal HCO_3^- reduced I_{sc} by 55 ± 8% ($n=5$) and removal of mucosal HCO_3^- reduced I_{sc} by only 7.6 ± 0.4% ($n=5$). In three bladders

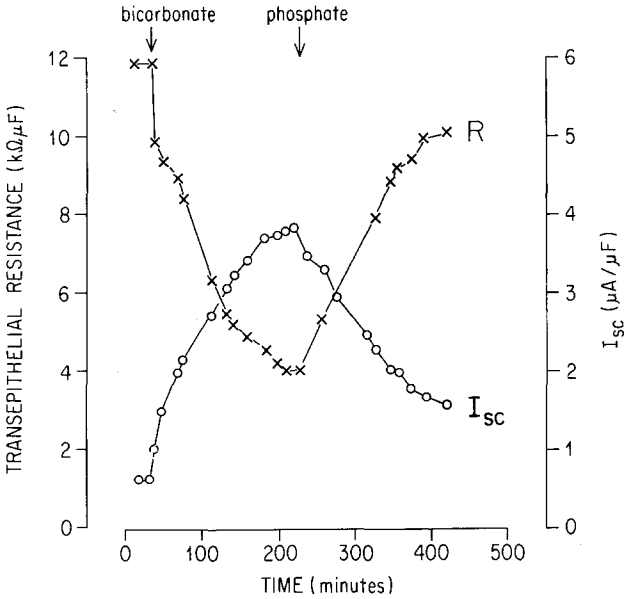


Fig. 6. Effect of HCO_3^- on I_{sc} (○) and R (×). Initially, the bladder was bathed in a solution without HCO_3^- (NaCl Ringer's solution, phosphate buffer). HCO_3^- was added at the first arrow ($NaCl-NaHCO_3$ Ringer's solution, $(HCO_3^-)=25$ mM) and removed at the second arrow. Note that HCO_3^- stimulates I_{sc} and decreased R

with low spontaneous I_{sc} ($<2\ \mu A/cm^2$) R decreased rather than increased on HCO_3^- removal from the serosal solution; and R increased again but not to the original value when serosal HCO_3^- was restored, with a half-time of 33 ± 5 ($n=3$) min. Probably the usual increase in R observed on HCO_3^- removal in high-current bladders involves the transport system, and the decrease observed in low-current bladders is an unmasked smaller effect [due to HCO_3^- removal causing nonspecific damage and shunt formation and thereby producing a shunt conductance (p. 17)].

In gallbladder, HCO_3^- similarly stimulates Na^+ transport, for reasons presently unclear (Diamond, 1964; Wheeler, Ross & King, 1969; Martin & Murphy, 1974). Martin and Murphy suggested that carbamyl phosphate is formed by HCO_3^- plus ATP and serves as an energy source for the pump. From studies of effects of carbonic anhydrase inhibitors Wheeler *et al.*, (1969) suggested that this enzyme affects a common transport mechanism for NaCl and $NaHCO_3$ in gallbladder and is also essential for optimal cellular functions other than ion transport. The decrease in I_{sc} on HCO_3^- removal in rabbit urinary bladder might at first suggest HCO_3^- transport from serosa to mucosa. However, tracer flux measurements will show I_{sc} to be due entirely to Na^+ transport.

2. *Effect of amiloride on* I_{sc}. Amiloride, a pyrazine diuretic, inhibits Na^+

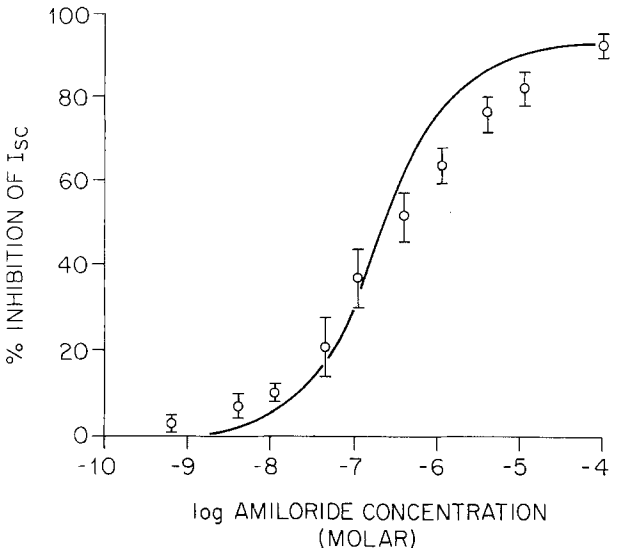


Fig. 7. Dose-response curve for inhibitory effect of amiloride, added to the mucosal solution at the indicated concentration, on normalized I_{sc} (ratio of I_{sc} with amiloride to I_{sc} before amiloride). Vertical bars indicate SEM for six experiments. The solid curve is the best fit to Michaelis-Menten kinetics, which do not fit the data well

transport in renal distal tubule (Baer, Jones, Spitzer & Russo, 1967), toad bladder (Bentley, 1968), toad colon and skin (Crabbé & Erlij, 1968; Erlij & Crabbé, 1968) frog skin (Salako & Smith, 1970), and rabbit colon (S. Schultz, *personal communication*). However, there is no so-called "leaky epithelium" (Frömter & Diamond, 1972) in which an effect has been demonstrated. In rabbit urinary bladder, amiloride reduced I_{sc} (*see* Fig. 7 for dose-response curve) and increased resistance, but only when added to the mucosal solution. The effect had a half-time of a few seconds, was complete within 15 sec, and was reversible when the mucosal surface was washed with 400 ml fresh solution. At 10^{-4} M amiloride increased R to values as high as 78,000 Ω μF [average, $43{,}000 \pm 4{,}250$ Ω μF ($n = 15$)]. The relation between the amiloride-dependent I_{sc} decrease and R increase, and the dependence of the magnitude of these amiloride effects on the spontaneous I_{sc}, will be presented in Figs. 15 and 16.

3. *Effects of hormones on* I_{sc}. The hormone aldosterone conserves body Na^+ by stimulating Na^+ absorption from toad bladder, renal distal tubule, sweat gland duct, salivary gland duct, colon and other organs (Edelman & Fimognari, 1968; Crabbé, 1972; *see* Sharp & Leaf, 1973 for a review). Added to the serosal surface of rabbit urinary bladder at 19 μM, aldosterone increased I_{sc} by $88 \pm 23\%$ ($n = 6$) and decreased R by

$35 \pm 6\%$ ($n=6$) after a lag of 40–90 min. The lag is similar to that observed for aldosterone effects on I_{sc} in other epithelia.

Antiduretic hormone (ADH) increases water permeability and Na^+ transport in renal collecting duct, toad urinary bladder and frog skin. In four experiments addition of 100 mU ADH (Parke-Davis Pittressin) to the serosal surface of rabbit urinary bladder had no effect on I_{sc} but caused a small decrease in R (by $22 \pm 8\%$) that could not be reversed by washing.

4. *Effects of metabolic inhibitors on* I_{sc}. Substitution of N_2 for O_2 reversibly caused a 90% decrease in I_{sc}, and an initial 20% increase followed by a 50% decrease in R. The effects of NaCN (1 mM) and of ouabain (1×10^{-4} M) were qualitatively the same as those of N_2 but were poorly reversible. The reason that HCO_3^-, amiloride, aldosterone and (initially) NaCN, N_2 and ouabain affect I_{sc} and R in opposite ways will be discussed in the following paper (Lewis *et al.*, 1976). The eventual decrease in R with N_2, NaCN, or ouabain is reminiscent of that seen on HCO_3^- removal in bladders with low I_{sc}. A possible explanation is that inhibition of ionic pumps caused cells to swell and lyse, creating non-specific damage and shunt pathways.

5. *ATPase activity.* ATPases activated by Na^+ and K^+ and inhibited by ouabain have been demonstrated in many cells and are believed to be part of the Na^+ transport system. In one experiment on a homogenate of stripped rabbit urinary bladder we measured an ATPase activity of 6.8 μmoles phosphate released per hr per mg protein. Of this activity, 2.1 μmoles/mg per hr, or 31% was inhibited by 1.4 mM ouabain. Ouabain-inhbited ATPase activities for other tissues summarized by Bonting (1970) range from 0.2 to 8.0 μmoles/mg per hr.

6. *Tracer fluxes.* (a) *^{22}Na.* Fig. 8 depicts the one-way tracer fluxes (J_{MS} = mucosa-to-serosa flux, J_{SM} = serosa-to-mucosa flux) in the short-circuited state, plotted as a function of the I_{sc}. J_{SM} showed no correlation with I_{sc}, R, or J_{MS}, and had an average value of 1.4×10^{-12} moles/cm^2 per sec ($\pm 0.7 \times 10^{-12}$, $n=19$). (Measured fluxes, like I_{sc} and R, were related to membrane capacitance, but have been recalculated to estimated apical membrane area on the basis $1\ \mu F \sim 1\ cm^2$, in order to calculate permeability coefficients in units cm/sec.) This flux, divided by bathing solution (Na^+), yields a Na^+ permeability $P_{Na} = 1.1 \times 10^{-8}$ cm/sec. In contrast, J_{MS} varied linearly with I_{sc}. The ratio J_{MS}/J_{SM} was on the average 30 ± 7 ($n=28$). $(J_{MS} - J_{SM})/I_{sc}$ equalled 1.03 ± 0.08 ($n=28$). Thus, the short-circuit current equalled the net Na^+ flux within experimental error. In the open-circuited state J_{SM} still showed no correlation with R or spontaneous I_{sc}, J_{MS} increased approximately linearly with tissue conductance

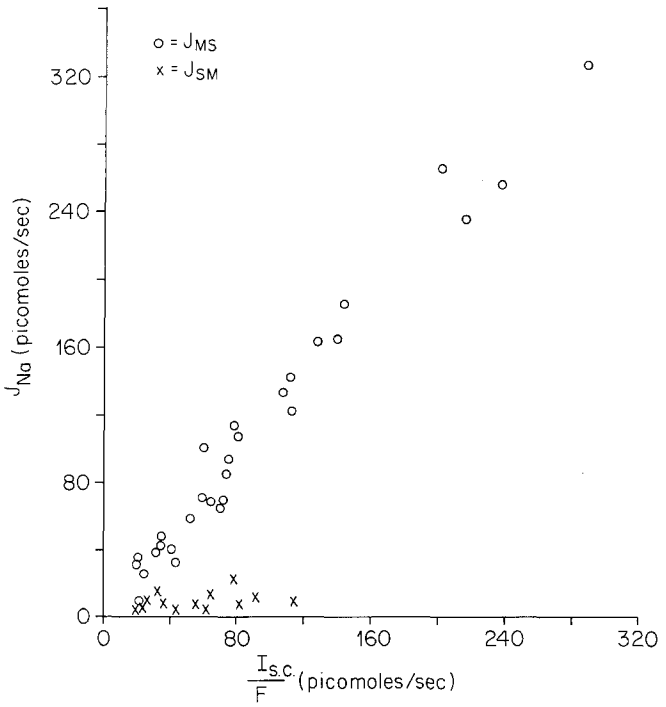


Fig. 8. Relation between one-way tracer fluxes of ^{22}Na in the short-circuited state (ordinate) and total net flux of ions calculated as I_{sc}/F (abscissa). o, mucosa-to-serosa flux J_{MS}; ×, serosa-to-mucosa flux J_{SM}. Because of low flux values, J_{MS} and J_{SM} could not be measured in the same bladder. Each point represents one 1/2-hr flux determination. A wide range of I_{sc} values was obtained by manipulating the rabbits' diet (p. 22) or (in a few cases) by adding amiloride. Note that variation in J_{MS} but not in J_{SM} is closely correlated with variation in I_{sc}

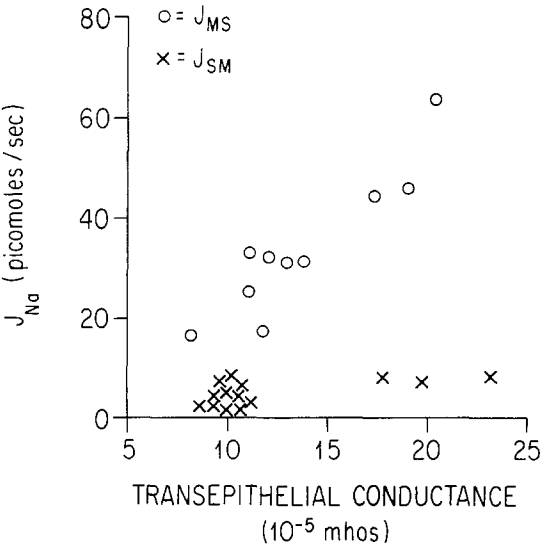
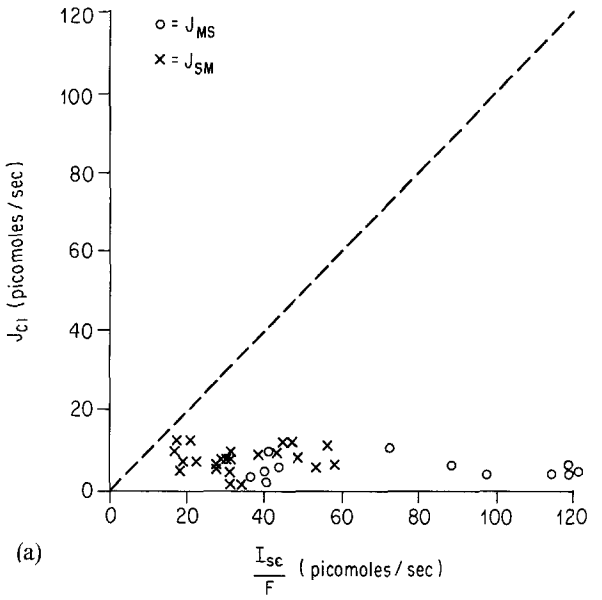


Fig. 9. One-way tracer fluxes of ^{22}Na on open circuit (o, J_{MS}; ×, J_{SM}) as a function of transepithelial conductance. Note that variation in J_{MS} but not in J_{SM} is closely correlated with spontaneous variation in G. Since G and I_{sc} are closely correlated (Fig. 16), this Figure means that the $J-I_{sc}$ correlations depicted on short circuit in Fig. 8 also apply on open circuit



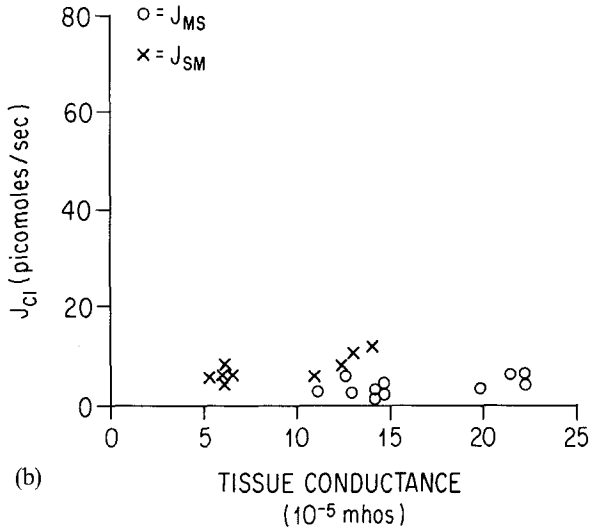


Fig. 10. (*a*) As Fig. 8, but for ^{36}Cl: relation between one-way tracer fluxes of ^{36}Cl in the short-circuited state (o, J_{MS}; ×, J_{SM}) and total net flux of ions I_{sc}/F. In contrast to the results for Na^+ depicted in Fig. 8, J_{MS} equals J_{SM}, neither flux varies with net transport rate, and net flux of Cl^- is zero. The broken line is the line of identity. (*b*) As Fig. 9, but for ^{36}Cl: one-way tracer fluxes of ^{36}Cl on open circuit (o, J_{MS}; x, J_{SM}) as a function of transepithelial conductance. Note that both J_{MS} and J_{SM} are independent of the transepithelial conductance

G ($=1/R$), and $(J_{MS}-J_{SM})/I_{sc}$ equalled 0.75 ± 0.11 (Fig. 9), where I_{sc} still refers to short-circuit current but where J_{MS} and J_{SM} now refer to open-circuit fluxes measured in the same bladder.

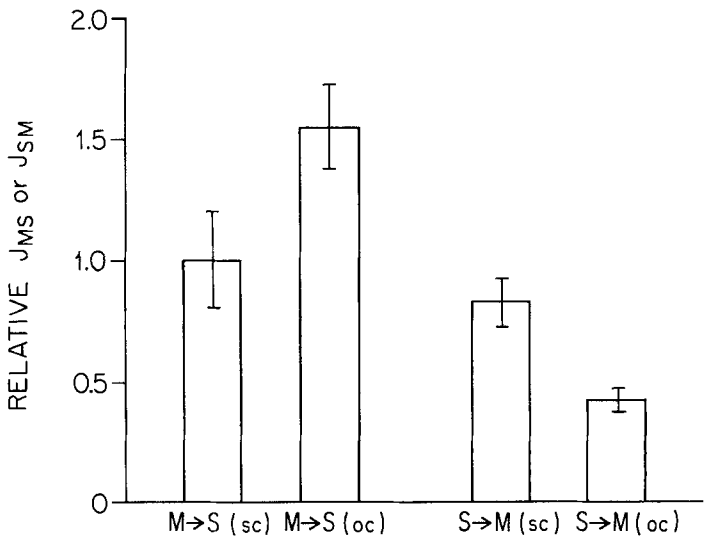


Fig. 11. One-way ^{36}Cl fluxes J_{MS} or J_{SM}, in short-circuited state (sc) or on open circuit (oc), divided by average value of J_{MS} in short-circuited state. Average values (height of bar) and SEM (vertical) error bars) are for nine measurements. Note that J_{MS} (sc)$=J_{SM}$ (sc) (i.e., net Cl^- flux is zero at short circuit); and that going from short circuit to open circuit increases J_{MS} but decreases J_{SM}, due to the serosa-positive voltage (average value, 40 mV)

(b) $^{36}Cl^-$. The net flux of Na^+ in the open-circuited state must be accompanied by net absorption of an anion, or by net secretion of a cation, or by both. The first possibility was tested by measuring J_{MS} and J_{SM} of ^{36}Cl on open circuit and short circuit. As shown by Fig. 10*a* and *b*, the one-way fluxes on short circuit did not correlate with I_{sc} or with G, and J_{MS} $(2.5\pm0.5)\times10^{-12}$ moles/cm^2 per sec; $n=8$) did not differ significantly from J_{SM} $[(2.0\pm0.2)\times10^{-12}$ moles/cm^2 per sec; $n=10]$ within the large standard errors of measuring these very low fluxes. Thus, it appears that there is no active transport of Cl^-. On open circuit, J_{MS} increased slightly and J_{SM} decreased slightly with G (the reason is that the serosa-positive spontaneous potential increased with G, because I_{sc} increased more rapidly than G). Fig. 11 illustrates that going from short circuit to open circuit increased J_{MS} but decreased J_{SM} (because of this p.d.). However, the net flux of Cl^- on open circuit, $J_{MS}-J_{SM}$, was on the average 2.8×10^{-12} moles/μF per sec, only 26% of the Na^+ net flux on open circuit, 10.6×10^{-12} moles/μF per sec. Thus, Cl^- is not the main counterion for Na^+ transport.

Two tests demonstrate that Cl^- fluxes obey the independence principle. First, if the principle is obeyed, the flux ratio for Cl^- should equal

$$J_{MS}/J_{SM}=[(Cl^-)_M/(Cl^-)_S]\exp[(-zF/RT)(V_S-V_M)] \qquad (2)$$

where $(Cl^-)_M$ and $(Cl^-)_S$ are the mucosal and serosal Cl^- concentrations, respectively, $(V_S - V_M)$ is the transbladder voltage, z is the valence (-1 for Cl^-), and F, R and T have their usual meanings. Average values on open circuit with identical solutions $[(Cl^-)_M = (Cl^-)_S]$ are $(V_S - V_M) = 40 \pm 2$ mV ($n = 38$), $J_{MS}/J_{SM} = 3.3 \pm 0.4$ ($n = 8$). For this voltage Eq. (2) predicts $J_{MS}/J_{SM} = 4.5 \pm 0.4$ in fair agreement with the measured value 3.3 ± 0.4. The second method is to compare calculated P_{Cl} values on short circuit and open circuit. From the one-way Cl^- flux on short circuit (average of all J_{MS} and J_{SM} determinations, $(2.2 \pm 0.3) \times 10^{-12}$ moles/cm^2 per sec ($n = 18$) and $(Cl^-) = 121$ mM, one calculates $P_{Cl} = J/(Cl^-) = 1.8 \pm 0.3 \times 10^{-8}$ cm/sec. On open circuit P_{Cl} may be calculated from the net flux J_{Cl} as

$$P_{Cl} = -zRTJ_{Cl}/(Cl^-)(V_S - V_M). \tag{3}$$

Substituting average values of $J_{Cl} = (2.8 \pm 1) \times 10^{-12}$ moles/cm^2 per sec ($n = 8$), $V_S - V_M = 40 \pm 2$ mV ($n = 20$) into Eq. (3) yields $P_{Cl} = 1.5 \pm 0.5 \times 10^{-8}$ cm/sec. The open-circuit and short-circuit P_{Cl} values, $(1.5 \pm 0.5) \times 10^{-8}$ and $(1.8 \pm 0.3) \times 10^{-8}$ cm/sec, are in approximate agreement, confirming that Cl^- fluxes obey the independence principle. A higher or lower value on open circuit than short circuit would have indicated single-file diffusion or exchange diffusion, respectively.

From J_{MS} or J_{SM} of Cl^-, J_{SM} of Na^+, and the concentrations of these ions we may calculate partial conductances, reflecting passive transepithelial diffusion, of 7.6×10^{-6} mho/cm^2 for Cl^- and 5.0×10^{-6} mho/cm^2 for Na^+. G_{Cl} and G_{Na} account for only 16 and 10%, respectively, of the total conductance in a bladder with low I_{sc} and G ($I_{sc} \sim 2.5$ μA/μF, total $G \sim 4.9 \times 10^{-5}$ mho/μF), and these values decrease to 7.6 and 5.0%, respectively, in a bladder with high I_{sc} (~ 12 μA/μF) and G ($\sim 1 \times 10^{-4}$ mho/μF). However, in a bladder with transport completely inhibited ($G \sim 1.4 \times 10^{-5}$ mho/cm^2), the passive fluxes of Na^+ and Cl^- account for 54 and 36%, respectively, of the total epithelial conductance.

Three conclusions can be drawn from these tracer fluxes of ^{22}Na and ^{36}Cl:

1. Na^+ accounts for all the short-circuit current and is the sole actively transported ion, unless active fluxes of two other ions fortuitously cancel. Cl^- is not actively transported.

2. Some ions other than Cl^- (presumably serosa-to-mucosa fluxes of K^+ and/or H^+, and/or a mucosa-to-serosa flux of HCO_3^-) are the main counterions for actively transported Na^+.

3. Much of the conductance in bladders with high transport rates is not accounted for by *transepithelial passive* fluxes of Na^+ and Cl^-. The

probable explanation is that current is carried across the apical cell membrane largely by Na^+, across the basolateral cell membrane by Cl^- or K^+ or both (Lewis *et al.*, 1976) and that our current pulses are much too small (<1 μA) and brief (1 sec) to alter intracellular ion concentrations.

Effect of Diet on I_{sc} and R

Under physiological conditions aldosterone release appears to be stimulated by restricted Na^+ intake and to be inhibited by Na^+ loading. In order to determine whether the aldosterone stimulation of I_{sc} observed *in vitro* is physiologically significant, we therefore attempted to vary natural release of aldosterone by varying dietary intake of Na^+. Five rabbits each were placed for five days on a high-Na^+ diet, normal diet, or low-Na^+ diet. (Na^+) and (K^+) levels in urine (collected by a metabolic tray) and in plasma were monitored daily, and at the end of this period the animals were sacrificed, their bladders excised, and I_{sc} determined. There was no statistically significant change in plasma (Na^+) and (K^+). Table 1 shows that with decreasing availability of dietary Na^+ the urine (K^+)/(Na^+) ratio increased (implying high aldosterone levels: Bartter, 1956), I_{sc} increased (*see* Fig. 12), and R decreased. The changes in I_{sc} and R are qualitatively similar to those observed *in vitro* after aldosterone.

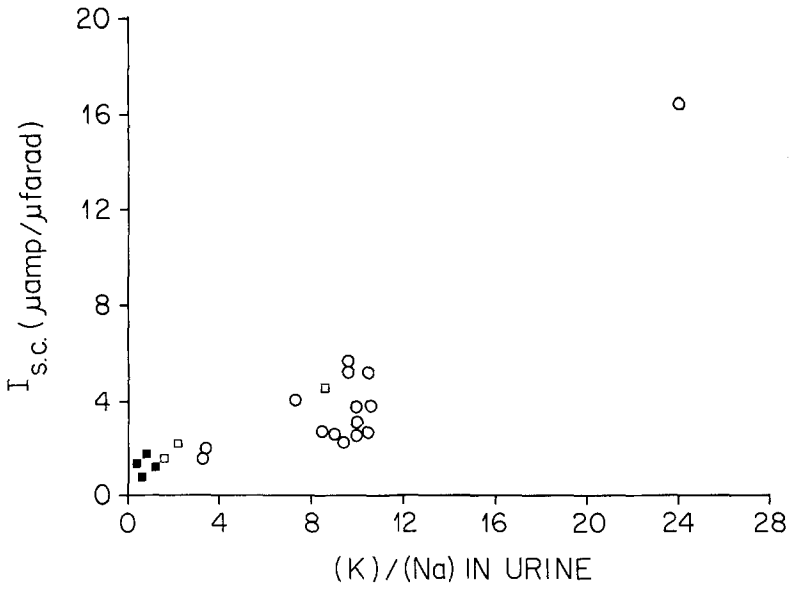


Fig. 12. Dependence of I_{sc} in excised bladders on urine (K^+)/(Na^+) ratio in the live rabbit. This ratio is correlated with circulating aldosterone levels. The rabbits' diets were high-Na^+ (■), normal (□), or low-Na^+ (○). Note that restricted Na^+ intake stimulates aldosterone release (as judged by high (K^+)/(Na^+) ratios) and stimulates I_{sc}

Table 1. Effect of diet on rabbit bladder and urine

Parameter	Diet		
	high-Na^+	normal	low-Na^+
Urine (K^+), mM	186±9	250±130	149±21
Urine (Na^+), mM	310±45	69±30	16±3
Urine (K^+)/(Na^+)	0.7±0.1	4.4±2.1	10.9±1.5
I_{sc}, μA/μF	1.6±0.3	2.2±1.4	5.1±1.3
R, Ω μF	23,000±2,300	16,700±4,000	12,700±1,300

Rabbits were kept on the indicated diet for five days. At the end of this time urine (K^+) and (Na^+) were determined; the animals were sacrificed, their bladders excised, and I_{sc} and R determined. Number of animals was 5, 3 and 10 on high-Na^+, normal, and low-Na^+ diet, respectively. The high-Na^+ diet consisted of 100 mM NaCl drinking water, Na^+ content in food 0.2 mequiv/g; normal diet, the same food but no NaCl in the drinking water; low-Na^+ diet, 100 mM KCl drinking water, food virtually Na^+-free.

Effect of pH on I_{sc} and R

Since the pH of urine varies physiologically from about 4.4 to 8, the effect of pH in the mucosal and serosal solutions separately on I_{sc} was studied.

As illustrated in Fig. 13, I_{sc} decreased with decreasing serosal pH: compared to pH 7.4, pH 8.4 stimulated I_{sc} by 15%, and pH 5.8 inhibited by 42%. Schoeffeniels (1955) reported qualitatively similar results for effects of serosal pH on I_{sc} in frog skin: 25% stimulation at pH 8.2, 95% inhibition at pH 5.3. If we assume that the $Na^+ - K^+$-activated ATPase is located on the basolateral (serosal-facing) cell membrane, these results might be explained in part by Bonting's (1970) finding that this enzyme has a pH optimum at 7.5 and that pH 5.5 decreases its activity by 60%.

Fig. 13 also shows that lowering mucosal pH from 7.4 to 4.3 has little effect (I_{sc} decreases by 11%, R decreases by 15%), but that a further lowering from 4.3 to 3.1 reversibly stimulates I_{sc} by 87% while decreasing R by 88%. Effects on the mucosal surface of frog skin are qualitatively similar but quantitatively different: lowering pH from 5.2 to 3.2 inhibits I_{sc} by 85%, and lowering from 3.2 to 2.2 stimulates by 30%, while lowering from 7.4 to 5.2 has no effect (Schoeffeniels, 1955). Two explanations suggest themselves for the stimulation of I_{sc} by very low mucosal pH. First, H^+ permeability might be sufficiently high that H^+ diffusion down its concentration gradient contributes to I_{sc} at low pH. Second, since mucosal (Ca^{2+}) inhibits I_{sc} (*see next section*), perhaps by blocking Na^+ entry into the epithelial cells, H^+ might stimulate Na^+ entry by displacing Ca^{2+}.

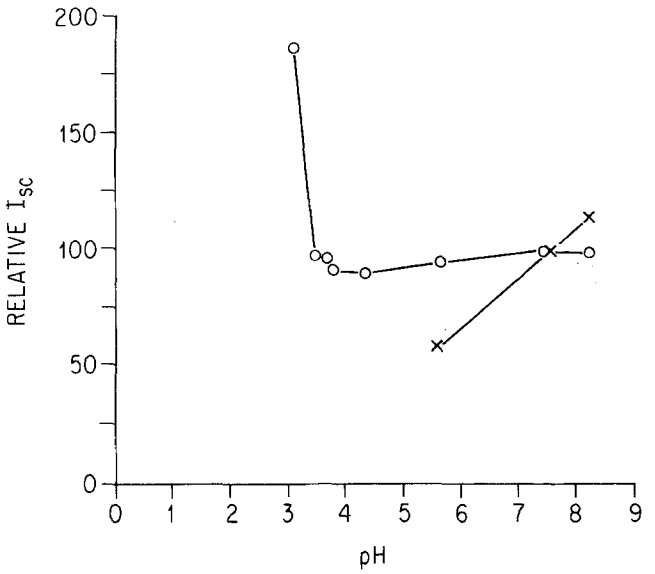


Fig. 13. Effect of pH on I_{sc}. ×, serosal pH varied (mean of two experiments), mucosal pH constant at 7.4. ○, mucosal pH varied (mean of three experiments), serosal pH constant at 7.4. Ordinate, I_{sc} at test pH as percent of I_{sc} at pH 7.4. For test pH values of 5.8 and higher, I_{sc} at the test pH and at 7.4 was compared using the same buffer

Effect of Divalent Cations on I_{sc} and R

Ca^{2+} inhibits I_{sc} in frog skin (Curran & Gill, 1962) and reduces P_{Na} of gallbladder (Wright & Diamond, 1968) and squid axon (Adelman & Moore, 1962). Since (Ca^{2+}) in mammalian urine varies physiologically from 0.2 to 18 mM, the effect of Ca^{2+} on I_{sc} was tested by washing both mucosal and serosal solutions twice with NaCl Ringer's solution lacking Ca^{2+} and Mg^{2+}. Dilution of dye concentrations to 0.3% of the initial value suggested by analogy that this wash reduced (Ca^{2+}) to about 6 μM. As illustrated in Fig. 14, the wash increased I_{sc} by $150 \pm 41\%$ ($n=8$) and decreased R by $36 \pm 4\%$ ($n=8$) after 30 min. However, these do not represent the maximum effect, since I_{sc} was still increasing and R still decreasing 30 min after Ca^{2+} removal. This extra I_{sc} caused by Ca^{2+} removal was little affected by amiloride, in contrast to the inhibition that this agent otherwise caused, but as was also found true for toad urinary bladder (Cuthbert & Wong, 1972). Restoration of Ca^{2+} to the serosal solution had no effect on I_{sc} or R within 10 min. Restoration of Ca^{2+} to the mucosal solution restored I_{sc} and R to their original values with a half-time of *ca.* 1.5 sec. Thus, the Ca^{2+} effect operates at the mucosal surface alone.

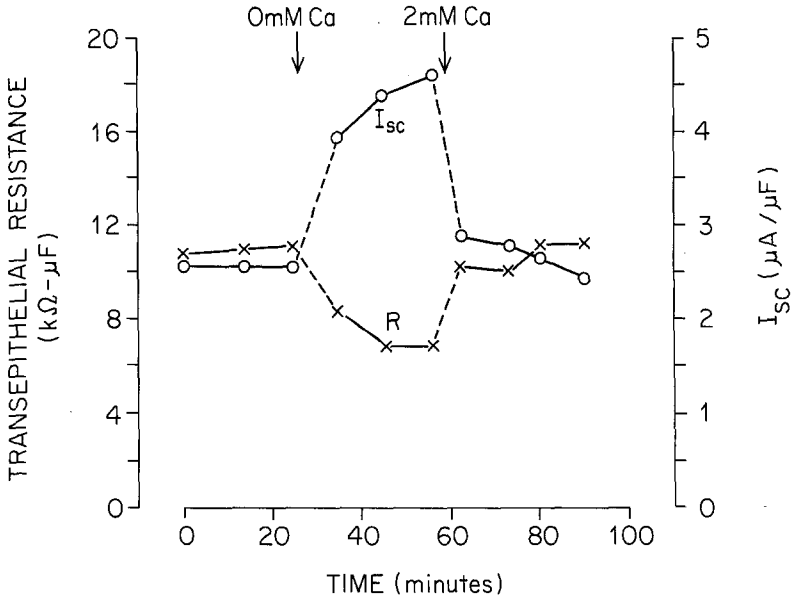


Fig. 14. Effect of Ca^{2+} on I_{sc} (o) and R (×). Initially, (Ca^{2+}) in both bathing solutions was 2 mM. At the first arrow Ca^{2+} was removed from both solutions (estimated actual concentration, 6 μM). At the second arrow 2 mM Ca^{2+} was restored to the mucosal solution only. Note that Ca^{2+} inhibits I_{sc} and increases R

The relative effects of the divalent cations Sr^{2+}, Ba^{2+}, Mg^{2+}, Ca^{2+} and Mn^{2+} at 2 mM on inhibition of I_{sc} were tested by removing Ca^{2+} from the mucosal solution, waiting 30 min, and then adding the test ion to the mucosal solution only. We expressed the percent inhibition of I_{sc} as $[I_{sc}(0\,Ca^{2+}) - I_{sc}(2\,X^{2+})]/[I_{sc}(0\,Ca^{2+}) - I_{sc}(2\,Ca^{2+} + 2\,Mg^{2+})]$ where I_{sc} ($2\,X^{2+}$) means the I_{sc} with 2 mM (X^{2+}) in the mucosal solution, etc. In two bladders the values of percent inhibition expressed in this way were Ca^{2+} 1.00 > Mn^{2+} 0.89 > Ba^{2+} 0.79 > Sr^{2+} 0.43 > Mg^{2+} 0.24; Ca^{2+} 0.93 > Mn^{2+} 0.78 > Sr^{2+} 0.35 > Mg^{2+} 0.13. Thus, Ca^{2+} is the most potent of the cations tested.

Sherry (1969) predicted possible potency sequences of alkaline-earth cations by comparing differences in cations' hydration energies with calculated differences in cations' electrostatic interaction energies with membrane negative sites. By varying site charge or spacing, Sherry showed that of the 24 permutations of the four alkaline-earth cations, only seven are expected as potency sequences from these considerations. Diamond and Wright (1969) showed that effects of these cations on most biological systems conform to these sequences. Our results add a further instance of agreement: the sequence $Ca^{2+} > Ba^{2+} > Sr^{2+} > Mg^{2+}$ is Sherry's sequence III. The sequence of alkaline-earth cation inhibitory effects on Na^+-

dependent O_2 uptake in toad urinary bladder is the same ($Ca^{2+} > Sr^{2+} > Mg^{2+}$; Ba^{2+} not tested), although the position of Mn^{2+} differs (Cuthbert & Wong, 1971). The sequence of divalent cation effects in reducing P_{Na}/P_{Cl} in rabbit gallbladder (Wright & Diamond, 1968) is essentially the same as that for inhibiting I_{sc} in rabbit urinary bladder. In addition, the cation 2,4,6-triaminopyridinium, which selectively abolishes cation conductance in the "leaky junctions" of rabbit gallbladder (Moreno, 1974), increases R and inhibits I_{sc} in rabbit urinary bladder (Lewis & Moreno, *unpublished observation*). Perhaps the molecular structure of the site controlling Na^+ entry in the apical cell membrane of tight epithelia is similar to that of the site controlling Na^+ permeation in the junctions of leaky epithelia.

Relation between I_{sc} and Membrane Conductance

We have now provided many examples of agents which affect I_{sc} and R oppositely (or I_{sc} and G in the same direction). Aldosterone *in vitro*, a low-Na^+ diet *in vivo*, and HCO_3^- stimulate I_{sc} while reducing R. Choline-for-Na^+ substitution, amiloride, a high-Na^+ diet *in vivo*, N_2 (initially), cyanide (initially), ouabain (initially), Ca^{2+}, low mucosal pH, and 2,4,6-triamino-pyridinium (TAP) all inhibit I_{sc} while increasing R.

We noticed a similar relation between spontaneous I_{sc}, which varied from about 1.5 to 20.5 μA/μF, and spontaneous R, which varied from about 26,000 to 7,000 ΩμF: bladders with high spontaneous I_{sc} had low R. These variations might reflect differences in aldosterone levels in rabbits before sacrifice.

We also noticed that there was much variation in the factors by which amiloride increased R and decreased I_{sc} of different bladders, and that these two effects of amiloride were related to each other and to the spontaneous I_{sc} and R values. The higher the spontaneous I_{sc} and the lower the spontaneous R, the greater were the factors by which amiloride increased R and decreased I_{sc} (Fig. 15). Similar correlations were observed between spontaneous I_{sc} and R and the magnitude of inhibitory or stimulatory effects of choline-for-Na^+ replacement (as was illustrated in Fig. 5), HCO_3^- (p. 14) and aldosterone.

Fig. 16 plots $G(=1/R)$ against I_{sc}, using results derived from the following sources: spontaneous variation; inhibition by amiloride, choline, Ca^{2+}, high-Na^+ diet and TAP; and stimulation by low Na^+ diet (possibly stimulated by aldosterone). All these results conform to the same striking pattern: G increases with I_{sc}. This means that a conductance pathway is an

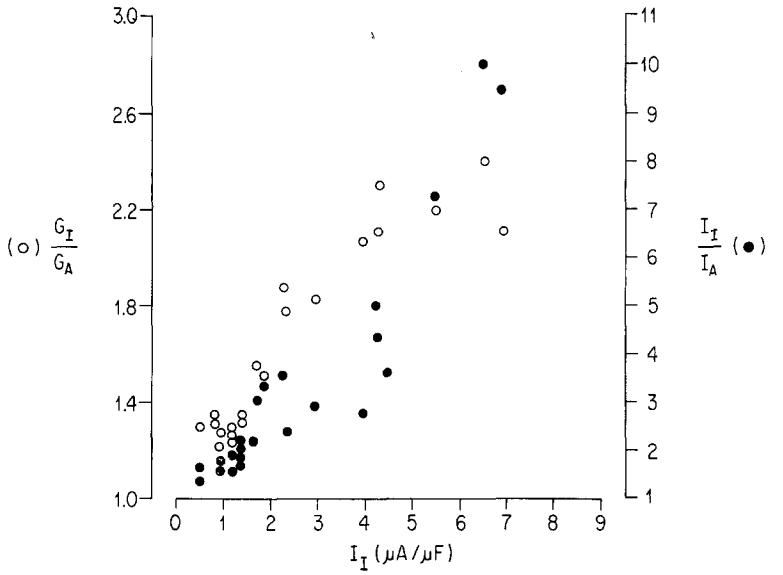


Fig. 15. Reciprocal of the factor by which 10^{-5} M amiloride decreases G (○) or I_{sc} (●) (ordinate), as a function of I_{sc} value before amiloride (abscissa). G_I/G_A, ratio of G before amiloride to G after amiloride. I_I/I_A, ratio of I_{sc} before amiloride to I_{sc} after amiloride. The larger the initial I_{sc}, the greater is the factor by which amiloride reduces G or I_{sc}

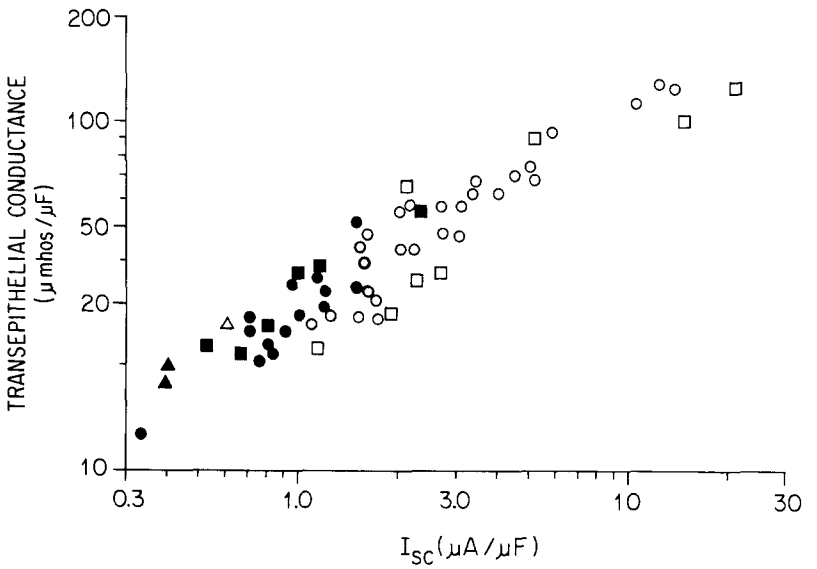


Fig. 16. Relation between conductance G and I_{sc}, both normalized to effective transepithelial capacitance. Symbols: animals on high-Na^+ (■), normal (□), or low-Na^+ (○) diets; bathing Na^+ replaced by choline (△); 10^{-4} M amiloride (●) or 10^{-3} M 2,4,6-triaminopyrimidine (▲) added to bathing solution. Note the close correlation between G and I_{sc}, defining the same quantitative relation under all these conditions

essential part of the transport system; and that all these agents affect the transport system while leaving this linkage between I_{sc} and G unchanged. The rapidity with which addition of amiloride or Ca^{2+} to the mucosal solution affects G (half-time ~ 1 sec), and the fact that amiloride, Ca^{2+}, and choline-for-Na^+ substitution are effective only from the mucosal surface, suggest strongly that this conductance pathway is in the apical cell membrane. Further evidence for this conclusion will emerge from microelectrode experiments (Lewis *et al.*, 1976).

Discussion

In this section we discuss in turn the similarities of rabbit urinary bladder to other tight epithelia, an interpretation of the G-*vs.*-I_{sc} relation,

Table 2. Comparison or transport properties

Property or agent	Rabbit urinary bladder		Toad urinary bladder		Frog skin		Frog urinary bladder	
	I_{sc}	R	I_{sc}	R	I_{sc}	R	I_{sc}	R
Na^+-free	−	+	−	+	−	+	−	+
Cl^--free	0	0			0	+	+	
HCO_3-free	−	+−			0	0	0	0
Ca^{2+}-free	+	−	[a]	[a]	+	−		
Amiloride	−	+	−	+	−	+	−	+
Aldosterone	+	−	+	−				
ADH	0	−	+	−	+	−		
Ouabain	−	+−	−		−	+		
Low mucosal pH	+	−			−	+		
Low serosal pH	−				−	+		
Na-K ATPase	Yes		Yes		Yes			
Na flux/I_{sc}	1:1		1:1		1:1			
K_m for Na	~44		~22		~22			
R_j	> 78,000 Ω μF		~12,000 Ω cm^2		> 40,000 Ω μF		> 33,000 Ω μF	

Rows 1–10: Effects of 10 agents on I_{sc} or transepithelial resistance (R), where known, are denoted by + (increase), − (decrease), 0 (no change), or +− (increase followed by decrease). The effects tested are: removal of Na^+, Cl^-, HCO_3^-, or Ca^{2+} from the bathing solutions; addition of amiloride, aldosterone, ADH, or ouabain; and low pH in mucosal or serosal bathing solution. Na reabsorption instead of I_{sc} was measured in renal collecting duct and distal tubule. P.d., which partly reflects I_{sc}, was measured in salivary duct. Row 11: "yes" means that Na−K-activated ATPase has been demonstrated in membrane fragments. Row 12: 1:1 means that net Na^+ flux approximately equals I_{sc}. Row 13: effective K_m for Na^+ transport, in mM. Row 14: junctional resistance. Main sources are: rabbit and frog bladders, frog skin, present study; frog skin, Ussing and Zerahn (1951); toad bladder, Ussing, Erlij and Lassen (1974); turtle bladder, Wilczewski and Brodsky (1975); renal collecting duct,

and the physiological significance of the Na^+ pump. Further discussion is postponed until the following paper (Lewis *et al.*, 1976).

Comparison with Other Tight Epithelia

Table 2 summarizes the results of this paper, by comparing transport properties of rabbit urinary bladder with those of two other well-studied tight epithelia, toad urinary bladder and frog skin. It is obvious that the transport mechanisms of these three tissues are very similar. In all three, junctional resistance is very high ($\geqq 12{,}000\,\Omega\,cm^2$, compared to 4–300 $\Omega\,cm^2$ for leaky epithelia (Frömter & Diamond, 1972). In all three, Na^+ accounts for most or all of the I_{sc}. Deviation of the I_{sc}-*vs.*-(Na^+) relation from Michaelis-Menten kinetics (Fig. 4) are observed in frog bladder and skin

of nine tight epithelia

Turtle urinary bladder		Renal collecting duct		Renal distal tubule		Salivary duct			Rabbit colon	
I_{sc}	R	J_{Na}	R	J_{Na}	R	p.d.	I_{sc}	R	I_{sc}	R
−	+	−		−		−			−	+
[b]	[b]			−		+			0	+
[c]	[c]	−[e]							0	+
−	+	−	+	−	+				−	+
+	−	+		+		+	+	−	+	−
		+	0							
−	+	−		−		−			−	0
							[f]			
[d]									Yes 1:1 42	
$>2{,}500\,\Omega\,cm^2$				$>600\,\Omega\,cm^2$		$>800\,\Omega\,cm^2$			>300	

Burg and Orloff (1973); renal distal tubule, Giebisch and Windhager (1973); salivary duct, Knauf and Frömter (1970); rabbit descending colon, S. Schultz (*personal communication*).

[a] Cells lyse.
[b] Cl is actively transported.
[c] HCO_3 is actively transported.
[d] $I_{sc} = Na^+$ flux $- Cl^-$ flux $- HCO_3^-$ flux.
[e] Gauged by effect on V_{sp}.
[f] Mucosal pH seems to regulate Na^+ transport.

Note that many agents affect I_{sc} and R in opposite directions in a given tissue; and that the various tissues often share similar properties.

(Lewis, Clausen & Diamond, 1975) as well as in rabbit bladder. The apparent K_m values for Na^+ transport are similar. Where tested, the effects of choline-for-Na^+ replacement, amiloride, aldosterone, ouabain, pH and Ca^{2+} on I_{sc} and R are parallel in the three tissues. In toad bladder (Saito & Essig, 1973) as in rabbit bladder, J_{MS} of ^{22}Na is linearly related to G, while J_{SM} is independent of G or I_{sc}.

Differences are that ADH stimulates I_{sc}, and that Cl^- conductance is significant, in frog skin and toad bladder but not in rabbit bladder. Table 2 also shows that six other tight epithelia (frog and turtle urinary bladders, renal collecting duct, renal distal tubule, salivary duct and rabbit descending colon) have similar properties, where these have been measured. The linkage between Na^+ transport and the aldosterone-stimulated, amiloride-inhibited conductance pathway in these tight epithelia will be discussed further in the following paper (Lewis *et al.*, 1976).

Interpretation of the G-vs.-I_{sc} Relation

A simple model permits one to extract from the *G-vs.-*I_{sc} relation (Fig. 16) estimates of limits or values for junctional conductance G_j and basolateral cell membrane conductance G_b. This method was developed independently by Higgins *et al.* (1975).

Consider the simplified electrical circuit of an epithelium depicted in Fig. 17. Transepithelial conductance G_t is given by

$$G_t = G_j + G_a G_b/(G_a + G_b). \tag{4}$$

We have seen that G_t increases with I_{sc}. From the experiments of this and the following paper we assume that this relation is due to an increase in G_a with I_{sc}; that G_j is independent of I_{sc}, and G_b is either independent of I_{sc} or else varies much more weakly than does G_a; and that G_b is larger than G_j. In the limits where I_{sc} and G_a are very low or very high, Eq. (4) approaches the lower asymptote $G_t \sim G_j$ (Region 1, Fig. 17) or the upper asymptote $G_t \sim G_b$ (region 3, Fig. 17), respectively.[1] In the intermediate

1 Note that a transport-independent conductance G_{al} in the apical membrane in parallel with the transport pathway G_a would have the same effect as a junctional conductance, i.e., to contribute to a lower asymptote. It is easily shown that the lower asymptote in this case becomes $G_{al}G_b/(G_{al}+G_b)$. Thus, this method cannot distinguish a junctional conductance from a transport-independent apical membrane conductance, and the lower asymptotes of Fig. 17 may overestimate the junctional conductance.

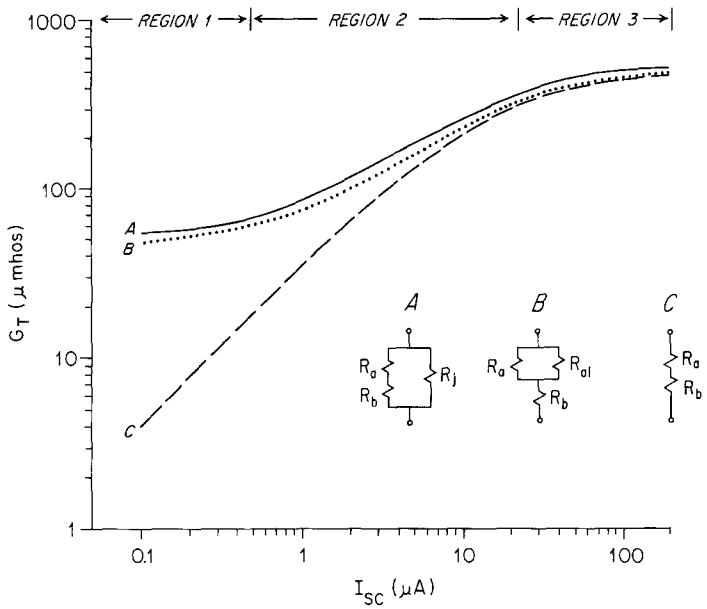


Fig. 17. Three simplified equivalent electrical circuits of an epithelial cell layer. *A*: junctional resistance $R_j = 1/G_j$, in parallel with the apical ($R_a = 1/G_a$) and basolateral ($R_b = 1/G_b$) cell membrane resistances in series. The transepithelial conductance G_t equals $G_j + G_a G_b/(G_a + G_b)$ for this network. *C*: as *A*, but $G_j = 0$. *B*: as *C*, but the apical membrane contains a transport-independent conductance or leak conductance G_{al} in parallel with a transport-dependent conductance G_a. The curves show the expected relation between G_t and I_{sc} for each circuit, on the assumption that $G_a = FI_{sc}/RT$. Solid curve, circuit *A*: $G_j = 50$ μmho, $G_b = 500$ μmho. Dotted curve, circuit *B*: $G_b = 500$ μmho, $G_{al} = 50$ μmho. Dashed curve, circuit *C*: $G_b = 500$ μmho. Note that for circuits *A* and *B*, G_t rises with increasing I_{sc} from a lower asymptote (region 1) of G_j (circuit *A*) or $G_{al}G_b/(G_{al} + G_b)$ (circuit *B*), through an intermediate range (region 2), to an upper asymptote of G_b (region 3, also seen for circuit *C*)

range (region 2, Fig. 17) G_t increases with I_{sc} and G_a. If experimental results were available for a sufficient range of I_{sc} to determine the asymtotes G_j and G_b, the dependence of G_a on I_{sc} for intermediate values of I_{sc} could also be extracted. Comparing Fig. 17 with the experimental G_t-*vs.*-I_{sc} relation of frog skin (Clausen & Lewis, *unpublished observations*), *Necturus* urinary bladder (Higgins *et al.*, 1975), and rabbit urinary bladder (this paper, Fig. 16) yields the following conclusions (Fig. 18):

In rabbit bladder we see suggestions of an upper asymptote but not a lower asymptote. We estimate $R_b \sim 7500\ \Omega\,\mu F$ (from the asymptotic $G_b \sim 1.33 \times 10^{-4}$ mho/μF $= 1/R_b$), R_j greater than 78,000 Ω μF (from the lowest *G* value at the left). In frog skin and *Necturus* bladder we see suggestions of a lower asymptote but not an upper asymptote, yielding estimates of $R_j \sim 24{,}000$ and 50,000 Ω cm^2, R_b less than 5000 or 1300 Ω cm^2, respectively.

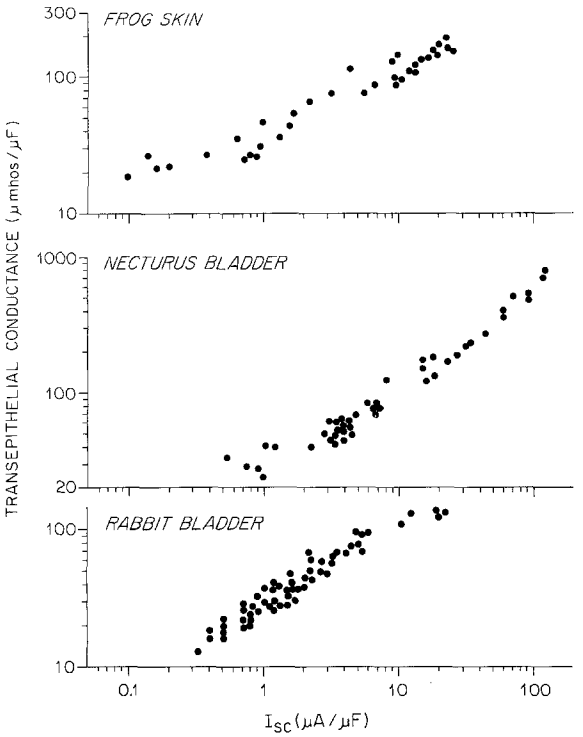


Fig. 18. Experimental relations between G_t and I_{sc} in frog skin (Clausen & Lewis, *unpublished observations*), *Necturus* urinary bladder (Higgins *et al.*, 1975) and rabbit urinary bladder (from Fig. 16 of this paper), for comparison with the theoretical relation of Fig. 17. There is a suggestion of an upper asymptote in rabbit bladder and of lower asymptotes in frog skin and *Necturus* bladder

Physiological Significance of the Na^+ Transport System in Mammalian Urinary Bladder

Our results have demonstrated that mammalian bladder is not an inert sac but actively reabsorbs Na^+. Cl^- will be absorbed passively, but this balances only part of the Na^+ absorption (p. 20), so that we must suspect passive, voltage-driven K^+ or H^+ excretion or HCO_3^- absorption. The Na^+ transport system is affected reversibly by variations in urinary pH or (Ca^{2+}) in the physiological range, and these variations may therefore be physiologically important. The Na^+ transport system is also affected by variation in dietary Na^+ intake, presumably mediated by aldosterone.[2] During Na^+ depletion aldosterone release causes the renal distal tubule

2 If the rabbits and dogs used in previous *in vivo* studies of bladder (cited on p. 2) were on diets with unrestricted Na^+ content, aldosterone levels and bladder pump activity would have been low, and the failure of these studies to discover the pump is not surprising.

to lower urine (Na^+), so that there will be a steep gradient in the bladder for Na^+ diffusion from blood to urine. Such diffusion would tend to dissipate the gradient established by the kidney. However, bladder Na^+ absorption is also maximal (due to aldosterone) during Na^+ depletion. The bladder transport system will tend to balance diffusional Na^+ influx and maintain urinary (Na^+) relatively constant. The bladder's very low electrical conductance except for that associated with the aldosterone-stimulated pathway, lower than that of almost any other epithelium, serves in addition to minimize diffusional Na^+ influx. (Figs. 8 and 9 demonstrated that Na^+ entry into the bladder via the aldosterone-stimulated pathway is undetectable.) Thus, a function of the bladder transport system is to prevent loss of Na^+ from the body during Na^+ depletion.

Could the bladder transport system significantly lower urinary (Na^+) if the Na^+ concentration in kidney urine entering the bladder were high? If we assume bladder volume to be 100 ml, area 104 cm^2 (from the formula for a sphere), and high transport rate 20 $\mu A/cm^2$ at (Na^+) = 136 mM or 10 $\mu A/cm^2$ at (Na^+) = 22 mM, then Na^+ reabsorption from bladder urine with (Na^+) = 22 mM would be 39 μmoles/hr. This transport rate would reduce (Na^+) by only 2%/hr. Thus, the pump of mammalian bladder cannot itself produce a urine of low (Na^+) from a high (Na^+) urine. It can only conserve a urine of low (Na^+), or else reduce (Na^+) further if the value is already low. For example, during an 8-hr overnight storage of urine with (Na^+) = 10 mM, the bladder would reabsorb only 13% of its Na^+ content at high pump rates.

What is the maximum gradient for Na^+ and Cl^- that the transport system can maintain? We estimate this limiting value for Na^+ by separately estimating the active Na^+ efflux and passive Na^+ influx as a function of Na^+ gradient (Fig. 19 above). The active efflux curve is obtained by multiplying Fig. 4 (relative active efflux as a function of concentration) times an assumed high pump rate of 20 $\mu A/cm^2$. To estimate the passive influx curve, we approximate the p.d. as RI_{sc}, where I_{sc} is estimated as a function of mucosal solution (Na^+) = $(Na^+)_m$ as in obtaining the efflux curve and where R as a function of I_{sc} is read from Fig. 17. The passive influx is then calculated as a function of $(Na)_m$ by the equation of Hodgkin and Katz (1949):

$$J_{sm} = (P_{Na} FV/RT)[(Na)_m - (Na)_s \exp(-FV/RT)]/[1 - \exp(-FV/RT)] \quad (5)$$

where P_{Na} is taken as 1.1×10^{-8} cm/sec from tracer flux measurements, V as RI_{sc}, and $(Na)_s$ as 137 mM, the value for rabbit plasma. Fig. 19 shows

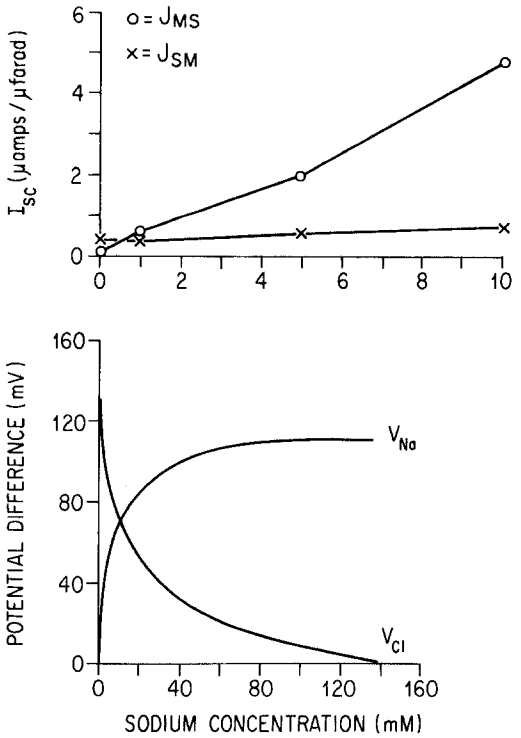


Fig. 19. Estimate of steady-state (Na^+) (above) and (Cl^-) (below) in rabbit bladder urine, assuming $I_{sc} = 20\,\mu A/\mu F$ and plasma (Na^+) = 140 mM and (Cl^-) = 137 mM. Abscissa: (Na^+) in urine (note different scales in upper and lower figures). Above: the Na^+ active efflux curve (symbols o; ordinate, $\mu A/\mu F$) is obtained by multiplying 20 $\mu A/\mu F$ times the relative efflux taken from Fig. 4, at the indicated abscissa urine (Na^+) value. The passive influx curve (×) as a function of urine (Na^+) is calculated from the p.d., (Na^+) gradient, and P_{Na}, as described in the text. The two curves intersect at 1 mM, the predicted steady-state (Na^+) in urine at this I_{sc} value after prolonged storage. Below: V_{Cl} (ordinate, mV), the Cl^- Nernst potential; V_{Na}, the p.d. generated by the active Na^+ pump. These two curves intersect at 13 mM, the predicted steady-state (Cl^-) in urine under the same conditions

that the influx and efflux curves cross at 1 mM, the expected equilibrium value of urine (Na^+) for a bladder with a pump rate of 20 $\mu A/cm^2$ at $(Na)_m \sim 136$ mM. The lowest urinary (Na^+) value actually measured in our rabbits was 5 mM. The order of magnitude agreement between 1 and 5 mM is satisfactory in view of the crudeness of our estimation procedure: we neglect contributions of diffusion potentials to V, assume I_{sc} to be independent of $(Na)_s$, and do not know whether the measured (Na^+) = 5 mM had reached equilibrium.

The maximum Cl^- gradient is estimated more readily, since we need only plot membrane voltage (estimated as in the preceding paragraph) and the Nernst equilibrium potential for Cl^- as a function of $(NaCl)_m$. For a

transport rate of 20 μA/μF the curves intersect at 13 mM (Fig. 19 below), the predicted equilibrium value of urinary (Cl^-) after indefinitely prolonged storage.

It is a pleasure to acknowledge our debt to Chris Clausen, Douglas Eaton, Austin Mircheff, Gabor Szabo, John Tormey and Ernest Wright, for discussion; to Douglas Eaton and Stanley Schultz for criticism of the manuscript; to Eberhard Frömter and Stanley Schultz for sharing unpublished results; and to Dr. H. D. Brown of Merck Sharp and Dohme for a generous gift of amiloride. This work was supported by grants GM 14772 from the National Institutes of Health, and by grant AM 17328 to the UCLA Center for Ulcer Research and Education.

Appendix

Effective Transepithelial Capacitance

Simon Lewis, Chris Clausen, and Jared Diamond

We consider the significance of effective transepithelial capacitance C_t in the light of an equivalent electrical circuit for epithelia, and we derive the conditions under which C_t is a measure of apical membrane area.

Lewis and Diamond (1975), and Cuthbert and Painter (1969*a*, *b*), Smith (1975), and Teorell (1946), have used membrane capacitance, calculated from the voltage response to a square current pulse, as a measure of the surface area of rabbit urinary bladder and frog skin, respectively. These authors used essentially the same electrical equivalent circuit to describe these epithelia (Fig. 20).

In this equivalent circuit, the apical membrane is modeled as a parallel RC combination (R_a and C_a, respectively; time constant $\tau_a = R_a C_a$). The basolateral membrane is similarly modeled (R_b and C_b, respectively; time constant $\tau_b = R_b C_b$). The tight junctions are modeled only as a parallel resistance, R_j. Tight junctional capacitance can be considered negligible for the following two reasons: membrane capacitance is directly proportional to membrane surface area, but the area of the tight junctions is minute compared to that of the apical or basolateral membranes; capacitance varies with membrane thickness, but the thickness of the tight junctions (*ca.* 1000 Å) is much greater than that of the cell membranes (*ca.* 80 Å). The small series resistance depicted in Fig. 20, R_s, corresponds to connective tissue, intracellular fluid and unstirred layers. It too will be neglected in the following discussion, since it contributes only a small, time-independent offset to the voltage response.

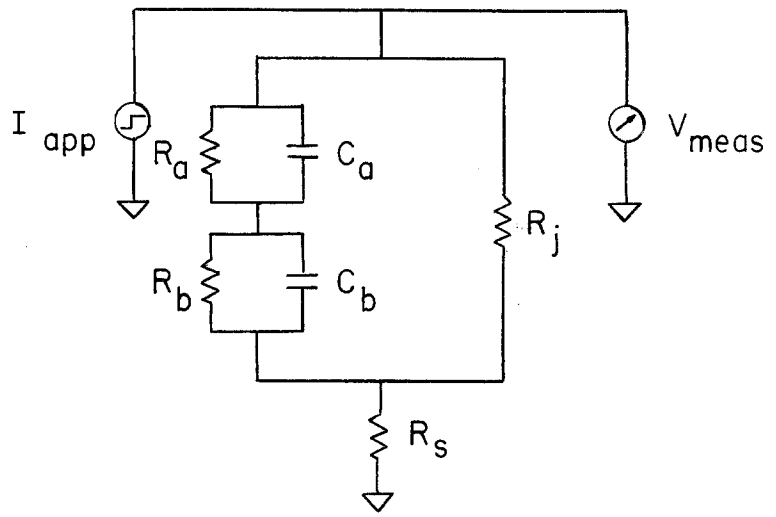


Fig. 20. Equivalent circuit of an epithelium, with resistors corresponding to the cell junctions (R_j), apical cell membrane (R_a), basolateral cell membrane (R_b), and series resistance (R_s), and with capacitors corresponding to the apical (C_a) and basolateral membranes (C_b). Transepithelial capacitance (C_t) may be calculated from the time-dependent voltage response (V_{meas}) to a square current pulse (I_{app})

By LaPlace transforms one can obtain the following expression for the voltage response, V_{meas}, as a function of time, given a square pulse of applied current, I_{app}:

$$V_{meas} = I_{app} R_t (1 - a_1 e^{-t/\tau_1} - a_2 e^{-t/\tau_2}) \qquad \text{(A.1)}$$

where R_t is the total DC resistance of the circuit ($R_t = R_j(R_a + R_b)/(R_a + R_b + R_j)$), and the parameters a_1, a_2, τ_1 and τ_2 are functions of the circuit elements. It should be emphasized that τ_1 and τ_2, the two time constants of the voltage response, are *not* generally equal to τ_a and τ_b (the apical and basolateral membrane time constants); τ_1 and τ_2 are functions of all the circuit elements.

Fourier analysis of V_{meas} without further measurements as employed by Teorell (1946), Cuthbert and Painter (1969*a*, *b*), and Smith (1975), is, in practice, not adequate to determine the circuit parameters of Fig. 20. Instead, it is convenient to measure the time τ_t required for V_{meas} to reach 63% $(1 - e^{-1})$ of its maximum (steady-state) value. Defining "effective transepithelial capacitance" as $C_t = \tau_t / R_t$, we now investigate the conditions under which $C_t \sim C_a$.

For various choices of values of circuit elements, we calculated τ_t and hence C_t from V_{meas}, and computed V_{meas} in turn from Eq. A.1). We held R_b constant at 7000 Ω, C_a 1 μF, and C_b 3 μF, the values in rabbit urinary

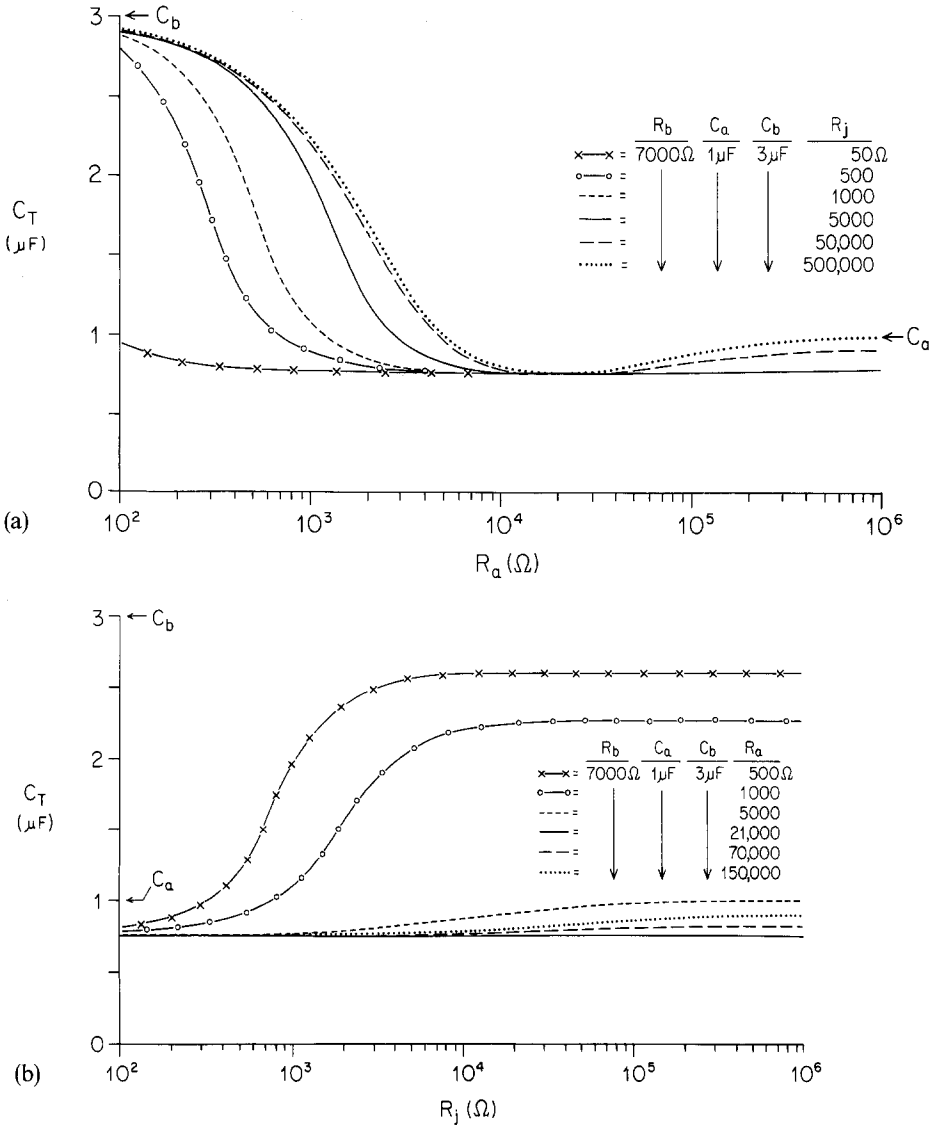


Fig. 21. (*a*) Effective transepithelial capacitance (C_t) calculated from Eq. (A.1), as a function of apical cell membrane resistance (R_a), for the various values of junctional resistance (R_j) given in the Figure inset. R_b was assigned a value of 7000 Ω, C_a 1 μF, and C_b 3 μF. In the right-hand part of the Figure the curves − × − × −, − o − o −, and ------- coincide with the curve ——— and are omitted. (*b*) Effective transepithelial capacitance, calculated from Eq. (A.1), as a function of junctional resistance (R_j), for the various values of apical resistance (R_a) given in the Figure inset. R_b, C_a and C_b were assigned the same values as in part *a*

bladder (Lewis *et al.*, 1976). Fig. 21*a* displays C_t as a function of R_a for different values of R_j, while Fig. 21*b* displays C_t as a function of R_j for different values of R_a. These Figures yield the following conclusions:

1. If $R_j \gg R_a, R_b$, then $C_t \to C_a$ for $\tau_a \gg \tau_b$, and $C_t \to C_b$ for $\tau_b \gg \tau_a$, as

may be seen from curve (···) of Fig. 21*a* or from comparison of the various asymptotic C_t values at the right of Fig. 21*b*. This case is discussed further in the text [pp. 8–9, Eq. (1)].

2. If $R_j \ll R_a, R_b$, then $C_t \to (C_a C_b)/(C_a + C_b)$ independently of R_a and R_b, as may be seen from curve (– × – × –) of Fig. 21*a* or from the convergent asymptotic values of all curves at the left of Fig. 21*b*.

3. When $\tau_a = \tau_b$, then $C_t \to (C_a C_b)/(C_a + C_b)$ independently of R_j, R_a or R_b, as seen from the convergence of all curves of Fig. 21*a* or from the horizontal curve (——) of Fig. 21*b*, for $R_a = 21{,}000\ \Omega$ ($\tau_a = \tau_b = 21{,}000\ \Omega\mu F = 21$ msec).

Thus, C_t may be used as a measure of apical or basolateral membrane area if R_j is very high and if $\tau_a \gg \tau_b$ or $\tau_b \gg \tau_a$, respectively. In rabbit urinary bladder the former condition may be obtained by applying amiloride to increase R_a, and R_j is spontaneously very high. This is why we used C_t in amiloride-treated bladders as a measure of apical membrane area. We have also used capacitance measurements to estimate C_a/C_b, as discussed by Lewis *et al.* (1976), especially Fig. 7*a*.

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