I. Introduction and Scope of Review

Maintenance of constant extracellular body fluid volumes and their osmotic and ionic concentrations depends on the regulated exchange of water and solutes across epithelia. These functions are accomplished by polarized epithelial cells, that is, the surface membranes of the two opposite sides of the cell containing different transport systems (147). Whether the epithelium forms a plane sheet or a tube, tight junctions (occluding zonules) form a continuous seal around the cell, providing a selective route for the exchange of molecules between the cells (10, 90, 320). The terminology of low-versus high-resistance epithelia was introduced for distinguishing between classes of epithelia of different paracellular conductance properties and functions in regulating ion and water content of body fluids (92). Low-resistance epithelia are usually specialized for bulk water transport in the absence of transepithelial osmotic gradients (“isosmotic water transport”). The occluding zonules are leaky, and the paracellular pathway constitutes a principal route for ion fluxes. High-resistance epithelia are maintaining or building up steep ion gradients between external and body fluid compartments. The occluding zonules constitute a tight seal restricting diffusion, and principal pathways of transepithelial fluxes are confined to cells.

Some high-resistance epithelia consist of more...
than one cell type specialized for the transport of ions from one side of the epithelium to the other. This review covers such heterocellular epithelia, and it discusses in more detail the role of a minority cell type, the mitochondria-rich (MR) cell, in transporting small ions across the epithelium. A major portion of this review is devoted to amphibian skin in which recent studies have shown that the MR cells constitute the route of transepithelial conductive chloride fluxes. The chloride conductance of the principal cells, which has been localized to the basolateral membrane, serves to regulate cell volume. Experiments leading to these extensions of the classic model of frog skin are discussed in detail. Mitochondria-rich cells of urinary bladder and kidney collecting duct of vertebrates are specialized for acid-base transport. It is a further aim of this review to discuss ion transport in distal renal epithelia to compare transport systems in MR cells of different high-resistance epithelia. This review concludes that MR cells of high-resistance epithelia, exhibiting a variety of transport functions such as acid and base secretion (α- and β-type, respectively) or passive and rheogenic active uptake of Cl⁻ (γ-type), are members of a common family of cells.

Recent reviews on the general aspects of ion transport by high-resistance epithelia cover amphibian skin (138, 144, 160, 167), the urinary bladder (150), and the collecting duct of the vertebrate kidney (19, 91, 212, 215, 274). Other reviews focus on epithelial cell volume regulation (317), ion channels in epithelial membranes (100, 198, 199, 268, 328), hormonal regulation of active transport (99, 186, 249), and acid-base transport (112, 182, 216, 291, 292, 303).

II. FUNCTIONAL ORGANIZATION OF AMPHIBIAN SKIN

A. Cell Types of Amphibian Epidermis

The amphibian epidermis is a multilayered epithelium (Fig. 1). The germinativum layer faces the basal lamina, followed by the stratum spinosum, the stratum granulosum, and the keratinized cells of the stratum corneum. The cells are held together by desmosomes, and they communicate with each other by gap junctions, which are assumed to form low-resistance pathways for the transport of water and small ions. The cells of the two outermost layers (stratum corneum and stratum granulosum) have tight junctions (43, 77, 281). Positioned in between the cells of the granulosum and spinosum layers are the flask-shaped MR cells. The neck of the MR cell is located in the granulosum layer and is linked to neighboring granulosum cells by tight junctions at the upper rim of the apex (25, 26, 337; Fig. 1). There are desmosomes between MR cells and principal cells, but they are fewer in number than between principal cells in general (337). On the apical membrane of MR cells facing the subcorneal space are microvillar ridges with a height of 0.5–1 μm. Besides the abundant mitochondria, this cell type is characterized by having a high density of small vesicles in the neck region. Lucent vesicles, as well as vesicles with moderately electron-dense contents, coated vesicles, and multivesicular bodies are found in the neck region. As in the principal cells, numerous vesicles arranged in rows or chains are also found at the base of the MR cells (337). Epidermal nerve fibers are usually adjacent to the MR cells (336).

The volume of the MR cell is ~500 μm³, and the apical membrane area is ~10 μm² (176). The density of MR cells varies among amphibian species, among individuals of the same species, and among different regions of the skin (63, 132, 338). In toads, MR cells, having an average density of 10⁶/cm², occupy 45 nl/cm² (338). This is <2% of the epidermal cell volume of 3,500 nl/cm². Likewise, the apical membrane area of the MR cells adds up to a small fraction (<1%, disregarding the area of microvilli) of the total epidermal surface area. Mitochondria-rich cells thus constitute a minority cell type of the epidermis, which explains why their role in transport of ions was overlooked for many years.

The so-called Merkel cells form another minority cell type of the epithelium (not shown in Fig. 1). They are much rarer than the MR cells and are identified by projections passing deep into neighboring principal cells and by their content of densely cored vesicles (228, 336). The Merkel cells occur just above the basal layer of the germinativum cells. Their function is still debated (110). It is not known whether Merkel cells play a role in the transport of ions through the epithelium.
The original model of frog skin (147) contained a single transporting compartment with a sodium-selective outward-facing membrane and a potassium-selective inward-facing membrane containing Na\(^+\)-K\(^+\) pumps (Fig. 2A). With this configuration of cation transport systems the model predicts that the skin transports Na\(^+\) in the inward direction, develops a transepithelial potential difference of mucosa-negative polarity, generates a sodium-carried short-circuit current, and maintains cation gradients between the intracellular and serosal compartments similar to other cells. It was further assumed that both membranes possessed passive Cl\(^-\) permeability. Numerous studies of solute transport across epithelia have shown that this first model of frog skin contained a general principle of how transporting epithelia are configured, that is, their polarized nature is due to an asymmetrical distribution of Na\(^+-\)K\(^+\) pumps and dissipative Na\(^+\) and K\(^+\) permeabilities.

The diagram in Figure 2B is an extension of the original model depicting the epithelium with two types of transporting compartments, principal cells and MR cells, in parallel with a paracellular route. Another extension of previous models is the presence in the inward facing membrane of a cotransport system on which the high intracellular Cl\(^-\) concentration of principal cells depends. What follows is a detailed discussion of studies leading to this model. First, a few general points are dealt with.

The cation pathways of the two transporting compartments are configured for active Na\(^+\) transport in an inward direction, according to the principle depicted in Figure 2A. The MR cells constitute a highly specialized pathway for transport of Cl\(^-\) across the epithelium, with no contributions from principal cells. The MR cells are depicted in two functional states. In Figure 2B, top, passive chloride permeabilities of both membranes allow Cl\(^-\) to flow along its transepithelial electrochemical potential gradient. Passive transport of Cl\(^-\) prevails in skins exposed to relatively large concentrations of Cl\(^-\) in the solution bathing the outside of the epithelium. Figure 2B, bottom, shows the model of the MR cell under conditions of active uptake of Cl\(^-\) (and inactivated apical chloride channels). The apical transport of Cl\(^-\) seems to be mediated by a Cl\(^-\)-HCO\(_3^\) exchange mechanism energized by an ATP-driven electrogenic proton pump (transporting H\(^+\) from the cell to the outside bath (70, 176a). This type of MR cell, the \(\gamma\)-type, is discussed in more detail in section VIII.

The skin also excretes body loads of acid and base (98, 322), and MR cells serve this function (62a, 112, 247). Necessarily, in such MR cells the proton-adenosinetriphosphatase (H\(^+\)-ATPase) and the anion-exchange system are configured in ways that are different from those depicted in Figure 2B. This point is discussed in more detail in section VIII.

The skin takes part in maintaining the K\(^+\) balance of the frog (89, 328). This may explain why, in some animals, the apical membrane contains a measurable K\(^+\) permeability (44, 71, 73, 235, 326, 327, 343–346) that mediates a significant outward flux of this ion (239). Some studies with frog skin also indicate the transport of K\(^+\) in an inward direction via an active mechanism (181, 241). These potassium-transporting functions of the skin are not considered in the models shown in Figure 2. Subepidermal glands constitute additional electrolyte-transporting compartments. The primary secretion of the acini is carried to the external side of the skin by ducts penetrating the epidermis (not shown in Fig. 1).

A type of \(\beta\)-adrenergically controlled gland secretes NaCl by means of active transport of Cl\(^-\) (16, 148, 223, 226, 300, 310). The transport function of the duct cells is poorly understood. The subepidermal glands of frog skin have served as an experimental model for the analysis of the ionic mechanism of bulk fluid transport in secretory epithelia (74, 75). A novel theory of "isotonic water transport" has been mathematically formulated (319), and some predictions have been successfully tested (242, 243, 319).

### B. Transport Models

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### C. Principal Cells of Epidermis Constitute a Functional Syncytium

Koefoed-Johnsen and Ussing (147) tentatively assumed that the Na\(^+\)-transporting compartment was identical to the germinativum cells of the epithelium.
Indirect evidence based on measurements of the intracellular electrical potential profile (220) and electron-microscopic analyses of epidermal cell junctions and localization of epidermal ATPase activity (76–78) led to an extension of the original model by suggesting that the cells of the epithelium communicate with one another through intercellular bridges of low electrical resistance. It follows that the outermost granulosum cells participate in transporting Na⁺ across the skin and that the Na⁺-selective membrane is identical to the apical membrane of the granulosum cells. These two aspects were verified by showing that the granulosum cells swell if the transepithelial active Na⁺ flux is increased (333) and that the distance covered by Na⁺ diffusing from the outside bulk solution to the Na⁺-selective membrane is no more than about the thickness of the cornified cell layer (94). The syncytium-like organization of the Na⁺-transporting compartment further implies that the inward-facing membrane containing the Na⁺-K⁺ pumps is identical with all membranes lining the intercellular spaces of the epithelium. In agreement with this notion, [³H]ouabain binding sites were shown to be located on these membranes with no binding sites on the outward-facing membranes of the outermost living cells nor on membranes of the cornified cells (224, 225). Low-frequency impedance analysis of the epidermis indicated the existence of junctional cell-to-cell passageways with a very low electrical resistance compared with the electrical resistance of the surface membranes of the cells (283). If Na⁺ (and other small ions) can flow from one cell to another via such low resistance bridges, then it is expected that elimination of the Na⁺ influx across the apical membrane results in a reduction of the intracellular Na⁺ concentration in all of the epidermal cell layers. This prediction was shown to hold in a study using the electron-microprobe technique for the measurement of intracellular cation concentrations in frog skin (260). With this technique it was also demonstrated that the germinativum cells derive all of their Na⁺ from the solution bathing the cornified side of the epithelium and that Na⁺ diffusing from the apical bathing solution to the germinativum cells passes the amiloride-blockable channels in the apical membrane of the granulosum cells. This elegant study provides the most compelling evidence for the hypothesis of a functional syncytium of the epidermal cells of frog skin.

Under different experimental conditions, the respective concentrations of Na⁺ and K⁺, at steady state, were found to be similar in all epithelial layers (50, 257, 260). Generally then, the gap junctions do not constitute a rate-limiting barrier for the transeellular active Na⁺ flux. However, under conditions of extremely stimulated active Na⁺ flux the diffusion resistance of the intercellular junctional membranes is revealed. For example, the active Na⁺ flux can be stimulated by clamping the potential of the apical bathing solution at a value that is positive with respect to the potential of the serosal bath. In such skins the outermost living cells swelled significantly, whereas the volume of the more deeply located cells decreased (333). Because the apical membrane of principal cells is tight to Cl⁻ (see sect. IIIc), this observation has an interesting implication. The swelling of the granulosum cells indicates that these cells gained a net amount of Na⁺ and Cl⁻. Furthermore, as the volume of the deeper cell layers decreased, these cells lost chloride and monovalent cations (e.g., Na⁺ was pumped out across the inward-facing membrane). Thus, during the non-steady-state period that elapsed between the onset of voltage clamping and the establishment of the new steady state, the inward-going transepithelial current carried by cations across the outward- and inward-facing membranes contained an intracellular component carried by Cl⁻ flowing via gap junctions in an outward direction between cells of the different strata. The resulting intraepithelial redistribution of diffusible ions suggests that the conductive Cl⁻ permeability of gap junctions in frog epidermis is larger than the conductive cation permeability. The antibiotic novobiocin, which increases apical Na⁺ permeability, resulted in the building up of transepithelial Na⁺ and Cl⁻ concentration gradients, with the concentration of both ions being highest in the outermost cell layer (256). Thus accumulation of Na⁺ in the outermost cell layer is associated with redistribution of transepithelial Cl⁻ resulting in cell volume expansion of the granulosum cells, confirming the observation by Voûte and Ussing (333).

Vasopressin (AVP) stimulation of the active Na⁺ flux in frog skin resulted in an increase in the Na⁺ concentration of all epithelial cell layers (261). The Na⁺ concentration of the outermost granulosum layer was slightly higher than that of the deeper layers. (In this study, the control skins showed a similar, but less steep, transepithelial Na⁺ gradient.) The gradient was eliminated if the Na⁺ concentration (CNa) of the outer bath was reduced (or if amiloride was present in the outer bath). Thus after AVP stimulation of the apical Na⁺ permeability, the resistance of gap junctions seems to contribute significantly to the overall resistance for active Na⁺ flow.

In the same study (261), removal of Na⁺ from the inner bath [Na⁺ replaced by arginine, choline, or tris(hydroxymethyl)aminomethane (Tris)] produced a significant loss of all three diffusible ions (Na⁺, K⁺, and Cl⁻). The net loss of intracellular ions (discussed in section IIIc) is caused by reversal of cotransport-transport across the basolateral membrane. This treatment also resulted in a Na⁺ concentration gradient with the Na⁺ concentration in the cell (CNa) being highest in the granulosum cells. As the short-circuit current decreased, the building up of a Na⁺ gradient was taken as evidence for the uncoupling of the cells rather than being associated with the transeellular active Na⁺ flux. It was speculated that uncoupling was caused by an increased intracellular concentration of diffusible Ca²⁺ or of protons (261). The normally low concentrations of these ions are maintained by Na⁺-dependent countertransport systems in the serosal membrane (62, 112, 113, 308).
D. Mitochondria-Rich Cells and Principal Cell Compartment Behave as Noncoupled Units

Under control conditions the intracellular steady-state cation concentrations follow the well-known pattern of other animal cells, that is, the Na⁺ concentration of the principal cell compartment and of the MR cells is ~10 mM, and their K⁺ concentration is 110-140 mM. However, a significant difference in C_Ci is maintained between the principal cells on the one hand and the MR cells on the other. Thus electron-microprobe analysis indicated a C_Ci of principal cells of 40-50 mM with insignificant variations among cells of the different layers and a C_Ci of MR cells of only ~15-25 mM (257, 261). With a method that uses the volume change of the two compartments in response to exposure of the epithelium to Cl⁻-free solutions, a similar set of C_Ci was estimated, that is, 40-50 mM in principal cell compartment (211, 314) and 20 mM in MR cells (176). Thus the two cellular units maintain different steady-state concentrations of the small Cl⁻.

Another reason for postulating that the two cellular units are not coupled to one another is that experimentally imposed changes of volume or ion composition of one of the two units do not result in similar changes of volume or ion composition of the other unit. 1) Ouabain applied to the serosal side of the epithelium resulted in the swelling of MR cells (176), whereas the volume of the principal cell compartment remained constant or decreased (211). 2) Under open-circuit conditions, the MR cell volume decreased in response to removal of Cl⁻ from the apical bath (286), with virtually no volume change of the principal cell compartment (313). 3) Dilution of the apical bathing solution with distilled water resulted in a considerable increase in MR cell volume (286). In an earlier study it was shown that the volume of the principal cell compartment remained constant after such a treatment (211). 4) Finally, stimulation of the active Na⁺ transport by vasopressin was associated with a significant increase in the intracellular Na⁺ concentration of the principal cells, whereas the Na⁺ concentration of MR cells did not change significantly (261).

These results indicate that MR cells and principal cells do not communicate with one another via low-resistance gap junctions.

III. TRANSEPITHELIAL IONIC CURRENTS AND THEIR VOLTAGE DEPENDENCE

A. Features of Steady-State Current-Voltage Relationship

The currents carried by Na⁺ (I_{Na}) and Cl⁻ (I_{Cl}) vary in a nonlinear fashion with the potential difference (V) imposed across the skin (Fig. 3). With NaCl Ringer-bathing both sides of the skin, the total current contains, for all practical purposes, two components (see Fig. 3A): I_{total} = I_{Na} + I_{Cl}. At short-circuit conditions (V = 0) the total current contains a large active Na⁺ component. Unlike frog skin (321), the short-circuit current of toad skin contains a small active Cl⁻ component (24). The most interesting point to be learned from the current-voltage (I-V) relations shown in Figure 3A is that amphibian epidermis has developed strongly rectifying pathways for both ions, as revealed by the fact that large net fluxes of Na⁺ and Cl⁻ are generated in the inward direction but not in the outward direction.

1. Chloride current rectification

With Cl⁻ being in equilibrium, toad skin (Bufo bufo) generates a small current carried by an inward flux of Cl⁻. Similar active Cl⁻ currents have also been described for other amphibian species [Leptodactylus ocellatus (341), B. viridis (139), and Rana pipiens (54)]. By comparing the current-voltage relationships depicted in Figure 3A and B, it can be seen that the strong outward-going rectification of I_{Cl} occurs independent of whether Na⁺ or K⁺ is present as the major cation of the outside bathing solution (170, 339). If the active sodium flux is eliminated with amiloride, the frog and toad skin exhibit a similar strong Cl⁻ current rectification (56, 158). All of these studies confirmed the general conclusion of the first study (24), that a large Cl⁻ conductance is available only for Cl⁻ flowing in the inward direction. This conclusion applies to steady-state currents. If a fast staircase-shaped voltage pulse is imposed across the epithelium of fully activated Cl⁻ conductance, then the resultant currents depict a linear function of V (Fig. 3C). This shows that the Cl⁻ conductance does not exhibit "instantaneous" rectification. The steady-state transepithelial conductance (G) is a continuous function of the voltage imposed across the skin (Fig. 4). With Cl⁻-free outside bathing solution, the conductance-voltage (G-V) relationships reveal ohmic-like behavior of the epithelium. The conclusions to be drawn from the relations in Figure 4 are that the chloride conductance (G_{Cl}) is found in a high conductive state in the physiological region of transepithelial potentials and is shut at V > 0 mV. The voltage thresholds of preparations from the two species are different. In skins of the European toad B. bufo the threshold normally is ~0 mV, i.e., G_{Cl} is almost eliminated at short-circuit conditions (Fig. 4A). In the skin of B. marinus a considerable Cl⁻ conductance prevails at V = 0 mV (Fig. 4B), as is also the case for preparations of frogs [R. temporaria and R. esculenta (158, 167)]. The general shape of the G_{Cl}-V curve is essentially the same for all animal species studied, indicating a common voltage-dependent conductance mechanism.

Control by voltage of G_{Cl} is further analyzed in section VI, and methods that have been used for shifting the position of the G_{Cl}-V curve along the voltage axis are discussed in section IVF.
2. Energy requirements of chloride currents

Figure 5 illustrates the dependence of the ratio of steady-state unidirectional Cl⁻ fluxes ($J_{Cl}^{in}/J_{Cl}^{out}$) on the quantity $V - V_{Cl}$. The full line is drawn according to the theoretical expression for simple passive fluxes of a monovalent anion (312, 296).

\[
J_{Cl}^{in}/J_{Cl}^{out} = \exp\left[-\frac{F(V - V_{Cl})}{RT}\right]
\]

where $R$ is the gas constant, $T$ is absolute temperature, $F$ is the Faraday constant, and $V_{Cl}$ is the transepithelial potential.

**FIG. 3. Steady-state current-voltage (I-V) relationship of toad skin and its major ionic components.** A: NaCl Ringer on both sides with large active Na⁺ current under short-circuit conditions ($I_{sc} = 0$). $V_{m}$ spontaneous skin potential ($I_{m} = 0$).[From Bruus et al. (24).] B: KCl Ringer on outside. [From Willumsen and Larsen (339).] C: I-V relation of a preparation held at holding potential ($V_{hold} = -97$ mV). Currents obtained within 50 ms so that Cl⁻ conductance had no time to inactivate. Apical Na⁺ channels were blocked with amiloride.[From Larsen and Kristensen (170).]

**FIG. 4. Steady-state conductance-voltage (G-V) curves.** Closed circles, Cl⁻ as major external anion; open circles, external Cl⁻ replaced with nonpermeant anion as indicated. A: skin of European toad R bufo [From Larsen and Simonsen (176).] B: skin of American toad B. marinus. [From Larsen et al. (176).]

**FIG. 5. Ratio of unidirectional steady-state Cl⁻ fluxes [Cl⁻ flux inward ($J_{Cl}^{in}$) and Cl⁻ flux outward ($J_{Cl}^{out}$)] as a function of externally applied driving force ($V - V_{Cl}$).** Line is theoretical relationship for electrodiffusive ion flows (see Eq. 2). [Data from Bruus et al. (24), Katz and Larsen (139), Dürr and Larsen (59), and Willumsen and Larsen (339).]
potential at which the Cl⁻ distribution across the epithelium is at thermodynamic equilibrium, i.e.,  
\[ V_{cl} = (RT/\lambda) \log\left(\frac{a_{cl}}{a_{cl}}\right), \]  
where \( a_{cl} \) and \( a_{cl} \) are the Cl⁻ activities in the outer and inner bathing solutions, respectively. After logarithmic transformation and with appropriate values of \( F, R, \) and \( T, \) Equation 1 takes the form 
\[ \log_{10} \left( \frac{J_{in}^{cl}}{J_{out}^{cl}} \right) = \left( -V - V_{cl} \right)/58.5 \] (2)

with \( V \) and \( V_{cl} \) in millivolts. According to the sign conventions defined, the driving force imposed on Cl⁻ is equivalent to \( -(V - V_{cl}) \) and is positive in the inward direction. The \( x \)-axis in Figure 5 is scaled according to \( V - V_{cl} \) for comparing the results discussed here with those depicted in Figure 3.

For an inwardly directed driving force imposed on Cl⁻ movement there is good agreement between theoretical and experimental flux ratios (Fig. 3, left). This means that the Cl⁻ conductance governing the large steady-state currents for \( V < 0 \) mV mediates passive fluxes of Cl⁻. When the driving force is in the outward direction, there is a significant deviation from the theoretical line. It is important that in this region the experimental flux ratio is close to unity and is independent of \( V - V_{cl} \). This finding holds whether the driving force is manipulated by varying \( V_{in} \) or \( V_{cl} \). It was concluded that a 1:1 chloride exchange pathway governs the steady-state chloride fluxes in this region (166). Additional support for this interpretation is discussed in section VIIA.

### B. Time Dependence of Chloride Currents

The above analysis of steady-state Cl⁻ fluxes and their energy requirements indicated that the passive Cl⁻ conductance prevailing in the physiological region of \( V (V < 0 \) mV) is being closed when \( V \) is brought to zero or is reversed (\( V > 0 \) mV). The transition of the passive Cl⁻ conductance from closed to activated state is a slow process, with time constants on the order of 10 s. The examples of Cl⁻ conductance activations shown in Figure 6A, left, were obtained by pulsing \( V \) in steps of 10 mV for 180 s from a holding value of \( V = 50 \) mV to the series of 11 potentials indicated at Figure 6A, top. Fully reversible conductance activations proceed along a sigmoidal course. The time-dependent currents were eliminated when Cl⁻ in the outside bath was replaced by gluconate (Fig. 6A, right), confirming that they are carried by an inward flow of Cl⁻.

The time it takes for the Cl⁻ conductance to reach steady state after an instantaneous shift of \( V \) depends on the voltage at which the activation takes place. This important property is conveniently illustrated by calculating the time \( t \) it requires to reach half-maximal conductance change. The \( t-V \) relation (Fig. 6B) is bell shaped with its maximum near \(-30 \) mV, i.e., in the \( V \) region where the \( G_{cl}-V \) relationship is steepest.

### C. Chloride Pathways of Principal Cells

The resemblance of the Cl⁻ conductance to excitable membrane cation systems and the fact that the activated Cl⁻ conductance was blocked by externally applied inhibitors of cellular transport systems, such as furosemide, phloretin, Diamox (159), and MK-196 (56), logarithmically associated the transport system with the apical membranes of the cells rather than with the tight junctions of the paracellular path. Studies of the Cl⁻ concentration and Cl⁻ pathways in the principal cell compartment are discussed next.

#### 1. Intracellular chloride concentration

Several methods have been used for estimating the intracellular Cl⁻ concentration of the principal cells. In the first study the intracellular Cl⁻ space was estimated by a method using the thickness of the epithelium as a measure of epithelial cell volume (211). When the external bathing solutions contained sulfate ions (practically impermeant to cell membranes), the height of the epithelium depended on the external tonicity according to the equation for an ideal osmometer with a constant osmotically inactive volume. It could be assumed, therefore, that cell volume changes are reflected in the change in height of the cells only, without any lateral cell expansion. With this assumption, the Cl⁻ concentration of the cell water was estimated from the increase in thickness of the epithelium in response to bilateral addition of Cl⁻ to skins pre-equilibrated in sulfate Ringer solution

\[ C_{cl} = \frac{(V_{in} - V_{out})}{(2V_{cl})} \] (3)

which further assumes that the cells are free of Cl⁻ in sulfate Ringer and that Cl⁻ is taken up together with a monovalent cation (K⁺). Here \( V_{in} \) and \( V_{out} \) are steady-state osmotic cell volumes at tonicities \( \Pi_{in} \) (Cl⁻ Ringer) and \( \Pi_{out} \) (SO₄²⁻ Ringer), respectively. With this method \( C_{cl} \) was found to be 40-50 mM (211, 314). From the values listed in Table 1, it can be seen that similar high concentrations have been reported in studies based on electron-microprobe analysis, washout experiments, and chemical analysis. This clear result has an important implication, i.e., practically all intracellular Cl⁻ of the principal cell compartment is associated with diffusible (osmotically active) pools.

With \( C_{cl} = 50 \) mM and mucosal Cl⁻ concentration (\( C_{cl}^{muc} \) = serosal Cl⁻ concentration (\( C_{cl}^{ser} \) = 115 mM) (Ringer solution on both sides of the epithelium), the Cl⁻ equilibrium potential is \( 21 \) mV. Under short-circuit conditions the intracellular potential is, on the average, \(-70 \) mV (229). Thus the concentration of Cl⁻ in principal cells is far above the equilibrium concentration. Over the past years this conclusion has been confirmed in studies using ion-selective microelectrodes. In short-
circuited preparations exposed to NaCl Ringer on both sides, the following mean values of Cl⁻ activities and intracellular potentials were reported: 18 mM, −78 mV (233); 22 mM, −57 mV (102), 15 mM, −90 mV (12), and 18 mM, −68 mV (339). Harvey and Kernan (114) reported an intracellular Cl⁻ activity of ~20 mM in frog skin under open-circuit conditions with an apical membrane potential of −38 mV (cell negative). Single (12, 233) as well as double-barreled microelectrodes (102, 114, 339) were employed on whole skins (12, 233) or isolated epithelia (102, 114, 339). Thus the intracellular Cl⁻ activities measured with ion-selective microelectrodes are independent of method and animal species.

An intracellular Cl⁻ activity of 20 mM indicates a Cl⁻ concentration of 26 mM, which is significantly lower than the value of ~50 mM obtained by the other methods (Table 1). The discrepancy between these two sets of values is too large to be trivial, and it remains to be explained. It is a fact, however, that the microelectrode studies unanimously confirm that the Cl⁻ concentration of the principal cells is significantly above the thermodynamic equilibrium concentration. For example, at short-circuit conditions the intracellular electrochemical potential of Cl⁻ is >2,800 J/mol above that of the bathing solutions, corresponding to an outward driving force of ≥30 mV.

2. Apical membrane of principal cells is not permeable to chloride

If the apical Na⁺ conductance is blocked with amiloride, then the principal cells hyperpolarize (116, 229).
the Cl- current increased by \(-90 \text{ PA/cm}^2\), but \(V_a\) and \(a_{\text{Cl}}\) for \(V = 40 \text{ mV}\) were \(-42 \text{ mM}\) and \(42 \text{ mM}\), respectively. After a shift to \(V = -100 \text{ mV}\) (fully activated Cl- conductance), the potential across the isolated epithelium was shifted from 40 mV (deactivated Cl- conductance) to -100 mV (fully activated Cl- conductance). Therefore, membrane potentials and the intracellular Cl- activity were monitored with a double-barreled microelectrode (339). In the example depicted in Figure 7, the transepithelial Cl- conductance increased by \(1.2 \pm 0.1 \text{ mS/cm}^2\), but \(F_{R_a}\) and \(a_{\text{Cl}}\) remained virtually constant and, as can be seen from Table 2, the shift of \(V\) from 40 to -100 mV changed, on the average, the driving force at the apical membrane by 101 mV (from 52 mV in the outward direction to 49 mV in the inward direction). At the basolateral membrane, the driving force changed by 37 mV (from 12 to 49 mV, both being in the direction from cell to serosal bath). During the subsequent minutes the transepithelial Cl- conductance increased by \(1.2 \pm 0.1 \text{ mS/cm}^2\). Table 2 also shows that the exchange of 36Cl- between tracer-loaded epithelia and the external solution is small (81%) or practically zero (233). Under short-circuit conditions, the transepithelial Cl- conductance is usually small, but in the negative region of the current-voltage curve, large passive Cl- currents flow through the skin (see Figs. 3 and 4). To investigate the Cl- conductance of the principal cells in skins with fully activated Cl- currents, the potential across the isolated epithelium was shifted from 40 mV (deactivated Cl- conductance) to -100 mV (fully activated Cl- conductance) while membrane potentials and the intracellular Cl- activity were monitored with a double-barreled microelectrode (339). In the example depicted in Figure 7, the steady-state apical membrane potential \((V^a)\) was \(-61 \text{ mV}\) and \(a^a_{\text{Cl}} = 42 \text{ mM}\) for \(V = 40 \text{ mV}\). After a shift to \(V = -100 \text{ mV}\) \((t = 2 \text{ min})\), \(V^a\) reversed its polarity to a new value of \(38 \text{ mV}\), indicating a fractional resistance of the apical membrane \((F_{R_a}) = 99/140 = 0.71\). Subsequently, the Cl- current increased by \(-90 \mu\text{A/cm}^2\), but \(V^a\) and \(a^a_{\text{Cl}}\) remained virtually constant and, as can be seen from the change in \(V^a\) after the return of \(V\) to 40 mV \((t = 4 \text{ min})\), \(F_{R_a}\) also remained constant. The driving force of the passive Cl- flow across the apical \((\Delta V^a_{\text{Cl}}/F)\) and the basolateral \((\Delta V^b_{\text{Cl}}/F)\) membrane, respectively, are defined by

\[
\Delta V^a_{\text{Cl}}/F = (RT/F) \log_e (a^a_{\text{Cl}}/a^a_{\text{Cl}}) + V^a
\]

\[
\Delta V^b_{\text{Cl}}/F = (RT/F) \log_e (a^a_{\text{Cl}}/a^b_{\text{Cl}}) - V^b
\]

where \(V^a\) and \(V^b\) are the apical and the basolateral membrane potentials, respectively. From the results in Table 2 it can be seen that the shift of \(V\) from 40 to -100 mV changed, on the average, the driving force at the apical membrane by 101 mV (from 52 mV in the outward direction to 49 mV in the inward direction). At the basolateral membrane, the driving force changed by 37 mV (from 12 to 49 mV, both being in the direction from cell to serosal bath). During the subsequent minutes the transepithelial Cl- conductance increased by \(1.2 \pm 0.1 \text{ mS/cm}^2\). As discussed in detail next, this steady-state distribution of Cl- is maintained by a Na+-dependent cotransport system in the basolateral membrane. By inspection of Figure 3B it can be seen that the transepithelial Cl- current is zero at \(V = -40 \text{ mV}\). In other words, at \(V = -40 \text{ mV}\) the apical membrane's passive Cl- permeability must be zero, otherwise asecretory Cl- current would have been detected. 2) Because \(F_{R_a}\) did not change and given the changes of the driving forces, the lack of change of \(a^a_{\text{Cl}}\) reflects that the apical membrane's Cl- permeability remains zero after full activation of the

### Table 1. Chloride concentrations or activities of principal cells of amphibian skin epithelium exposed to NaCl Ringer on both sides

<table>
<thead>
<tr>
<th>(C_{\text{Cl}}), mM</th>
<th>(a_{\text{Cl}}), mM</th>
<th>Species</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>49 ± 2</td>
<td>R. temporaria</td>
<td>Osmotic volume</td>
<td>211</td>
<td></td>
</tr>
<tr>
<td>49 ± 7*</td>
<td>R. temporaria</td>
<td>Electron microprobe</td>
<td>290</td>
<td></td>
</tr>
<tr>
<td>52 ± 6*</td>
<td>R. viridis</td>
<td>Electron microprobe</td>
<td>257</td>
<td></td>
</tr>
<tr>
<td>41 ± 4*</td>
<td>R. temporaria</td>
<td>Electron microprobe</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>44 ± 5*</td>
<td>R. temporaria</td>
<td>Electron microprobe</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>50 ± 3</td>
<td>R. temporaria</td>
<td>Chemical analysis</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>33.5 ± 1.6</td>
<td>R. ridibunda</td>
<td>Chemical analysis</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>61.2 ± 2.2</td>
<td>R. ridibunda</td>
<td>Chemical analysis</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>56.1 ± 3.9</td>
<td>R. ridibunda</td>
<td>Washout</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>47.0 ± 2.9</td>
<td>R. ridibunda</td>
<td>Washout</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>59.8 ± 3.8</td>
<td>R. ridibunda</td>
<td>Washout</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>18 ± 3</td>
<td>R. pipiens</td>
<td>Ion-selective microelectrodes</td>
<td>233</td>
<td></td>
</tr>
<tr>
<td>21.9 ± 1.5</td>
<td>R. ridibunda</td>
<td>Ion-selective microelectrodes</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>20.3 ± 1.6</td>
<td>R. temporaria</td>
<td>Ion-selective microelectrodes</td>
<td>114</td>
<td></td>
</tr>
<tr>
<td>15.5 ± 0.5</td>
<td>R. pipiens</td>
<td>Ion-selective microelectrodes</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>18.0 ± 4.4</td>
<td>R. buffo</td>
<td>Ion-selective microelectrodes</td>
<td>339</td>
<td></td>
</tr>
<tr>
<td>27.8 ± 5.4</td>
<td>R. ridibunda</td>
<td>Ion-selective microelectrodes</td>
<td>80</td>
<td></td>
</tr>
</tbody>
</table>

\(C_{\text{Cl}}\), cellular Cl- concentration; \(a_{\text{Cl}}\), cellular Cl- activity. *36.5 ± 5.0 mmol/kg wet mass; 25.4 ± 2.3 g dry mass/100 g. **39.9 ± 4.9 mmol/kg wet mass; 23.9 ± 2.2 g dry mass/100 g. *31.2 ± 3.1 mmol/kg wet mass; 23.3 ± 2.2 g dry mass/100 g. **33.6 ± 4.1 mmol/kg wet mass; 24.4 ± 2.7 g dry mass/100 g.
rrium concentration by a basolateral cotransport system driven by the Na\(^+\) concentration gradient was formulated on the basis of two types of experiments. Ferreira and Ferreira (81) found that removal of Na\(^+\) or Cl\(^-\) from the serosal bath produced a significant decrease in cell Cl\(^-\) together with a loss of water. In studies of cell volume regulation of frog skin epithelium, Ussing (313) showed that KCl-depleted epithelia were unable to regain KCl and recover their volume in the absence of sodium, or in the presence of furosemide, in the serosal bath. More recently it was shown that the recovery of cell volume required the presence also of potassium in the serosal bath. Furthermore, at steady state, the Cl\(^-\) space of the epithelium depends on the Cl\(^-\) concentration of the serosal bath as predicted if two Cl\(^-\) are transported per cycle (314). It was suggested, therefore, that the cotransport system operates with a stoichiometry of 1 Na\(^+\):1 K\(^+\):2 Cl\(^-\), that is, identical with the stoichiometry of the cotransporter in nonpolarized cells (101). In agreement with this hypothesis, it has been found that components of potassium and chloride fluxes across the basolateral membrane (of ouabain-treated preparations) are blocked by furosemide, and evidence was given that these flux components are carried by an electroneutral mechanism (35) that also carries sodium (34, 36). Measurements of cellular electrolyte concentrations using the microelectrode technique (102) and electron-microprobe analysis (48, 50) confirmed that the principal cells cannot maintain a high intracellular Cl\(^-\) concentration unless Na\(^+\), K\(^+\), and Cl\(^-\) all are present in the serosal bath. Rubidium, but not cesium, can replace potassium (48, 317), and lithium can replace sodium (317). Of the anions tested, only chloride and bromide are taken up by the cotransporter (49, 317).

If the stoichiometry of cotransport is considered, equilibrium prevails if the chemical potentials of the three ions fulfill the following condition

\[
RT \cdot \log a_{\text{Na}} + RT \cdot \log a_{\text{K}} + 2RT \cdot \log a_{\text{Cl}} = 0
\]

or, with similar activity coefficients in the two compartments

\[
C_{\text{Na}}^c \cdot C_{\text{K}}^c \cdot (C_{\text{Cl}})^2 = C_{\text{Na}}^i \cdot C_{\text{K}}^i \cdot (C_{\text{Cl}})^2
\]

Under control conditions, the left-hand side of Equation 6 was estimated to be only slightly smaller than its

### Table 2. Intracellular Cl\(^-\) activity and membrane potentials of principal cells of toad skin epithelium

<table>
<thead>
<tr>
<th>V, mV</th>
<th>(a_{\text{Cl}}^c), mM</th>
<th>(V^c), mV</th>
<th>(V^i), mV</th>
<th>(\Delta \phi_c/F), mV</th>
<th>(\Delta \phi_i/F), mV</th>
<th>(\Delta G_{\text{Cl}}), mS/cm²</th>
<th>(F_{G_c})</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>40 ± 4</td>
<td>71 ± 3</td>
<td>31 ± 3</td>
<td>52</td>
<td>12</td>
<td>0.69 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>-100*</td>
<td>42 ± 4</td>
<td>31 ± 6</td>
<td>-67 ± 6</td>
<td>49</td>
<td>49</td>
<td>1.2 ± 0.1</td>
<td>0.67 ± 0.03</td>
</tr>
<tr>
<td>-100+</td>
<td>41 ± 4</td>
<td>29 ± 6</td>
<td>-71 ± 6</td>
<td>47</td>
<td>47</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 15 impalements. Response to voltage activation of transepithelial Cl\(^-\) conductance \((G_{c})\). Driving forces for the passive Cl\(^-\) flows at 2 membranes were calculated according to Eq. 4, a and b. See text for definitions of abbreviations. * Before activation of \(G_{c}\). † After activation of \(G_{c}\). [Data from Willumsen and Larsen (339).]
right-hand side (314). Ussing (314) pointed out that this indicates that the cotransporter carries only a small Cl⁻ flux from bath to cells and that the leak flux of Cl⁻ is similarly low. Fernandez et al. (80) arrived at the same conclusion in a study of ionic movements across the basolateral membrane using ion-selective microelectrodes for the measurements of intracellular activities of Cl⁻ and Na⁺. The presence of a small leakage permeability for Cl⁻ was also indicated by experiments showing that neither the osmotic cell volume nor the intracellular potassium pool changed much when furosemide was added to the serosal NaCl Ringer (313). If, on the other hand, one of the three ions was omitted from the bath, the cell volume decreased, which is compatible with a net loss of Cl⁻ via the cotransporter (Eq. 6). In agreement with this interpretation it was recently shown that the loss of cell chloride to a Cl⁻-free bath is prevented if bumetanide is present in the serosal bath (48). Thus the cotransport system normally operates with a low but sufficient rate to counteract the backflux of Cl⁻ through a very small leakage permeability of the serosal membrane. This means that the high intracellular Cl⁻ concentration in the principal cells is maintained with a small energy expenditure.

Under isosmotic conditions of low intracellular Cl⁻ concentration (where cell volume is also decreased) the cotransport system becomes activated, but the activating factor is unknown (313, 317). Also the serosal membrane potential depolarizes, and its K⁺ selectivity decreases significantly, presumably due to closure of serosal membrane K⁺ channels (33). The Na⁺ taken up by the cotransport system is returned to the serosal bath in exchange with K⁺ by the Na⁺-K⁺ pump, with the net result being a cellular uptake of KCl and a restoration of cell volume. Cellular swelling activates the passive Cl⁻ permeability. Thus, by making the serosal solution hypertonic, cellular KCl is rapidly lost, and cell volume returns toward its control value (313). During recovery in normal Ringer solution, KCl is taken up again via the mechanisms explained. The cell volume responses were independent of the ion composition of the external solution, in agreement with the conclusion that the apical membrane of the principal cells is tight to Cl⁻ (313; see sect. IIIC2).

It can be concluded that in amphibian epidermis the cotransport system and the basolateral membrane's passive Cl⁻ permeability serve to regulate the intracellular Cl⁻ concentration (cell water volume).

4. Chloride conductance of basolateral membrane

Exposure of the serosal side of the epithelium to a hypertonic Ringer solution produced a rapid loss of cellular KCl, which could be prevented if the serosal solution contained the Cl⁻ channel blocker MK-196 (313, 315). The Cl⁻ conductance of the serosal membrane is also activated by depolarizing the serosal membrane, either by blocking the K⁺ channels with Ba²⁺ or by raising the K⁺ concentration in the serosal bath. In their activated state the Cl⁻ channels are permeable to small anions like Br⁻, I⁻, SCN⁻, and NO₃⁻ (316). With respect to their voltage dependence, their poor anion selectivity, and their pharmacology, the Cl⁻ channels of the basolateral membrane of the principal cells resemble the apical Cl⁻ channels in the MR cells (see sect. IV E).

5. Chloride-chloride and chloride-bicarbonate exchanges across basolateral membrane

Tracer studies have shown that a fairly fast exchange of ³⁶Cl⁻ takes place between the principal cell compartment and the serosal bath (81). As these fluxes were independent of membrane potential but could be inhibited by 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS), it was suggested that they are mediated by an electroneutral Cl⁻ self-exchange system of the red blood cell membrane type (301). It has also been shown that when Cl⁻ is removed from the serosal bath, cellular pH increases by 0.1-0.2 units. Because the alkalization could be inhibited by SITS, it was suggested that in the absence of serosal Cl⁻, cellular Cl⁻ exchanges with HCO₃⁻ of the serosal bath (31, 55). It is likely that it is a common reversible SITS [acid and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS)-sensitive anion-exchange system that operates in Cl⁻-Cl⁻ exchange and Cl⁻-HCO₃⁻ exchange modes. The exchange of cellular HCO₃⁻ for serosal Cl⁻ is stimulated at alkaline pH (112).

IV. IONIC PATHWAYS OF MITOCHONDRIA-RICH CELLS

The ionic pathways of MR cells of amphibian skin have been studied by several experimental methods. In this section the possible roles of the transport systems in mediating fluxes of Cl⁻ and Na⁺ across the epithelium are discussed.

A. Cell Density and Skin Conductance

Voûte and Meier (331) investigated the relationship between the current carried by Cl⁻ and the number of MR cells per millimeter section length of fixed epithelia stained with toluidine blue. It was shown that short-circuited epithelial preparations of R. esculenta exposed to KCl Ringer on the outside and K-glucuronate Ringer on the inside maintained inward fluxes of Cl⁻ that were linearly correlated with the number of MR cells. Microscopic examination of fixed and stained preparations indicated that the MR cells were swollen in contrast to principal cells of unaltered or slightly decreased volume. These findings were explained by assuming that the apical Cl⁻ conductance of the epithelium is confined to the apical membrane of MR cells. With the purpose of eliminating transepithelial sodium currents, potassium was used as the major cation of both bathing solutions.
Probably, however, the successful result of this study is due to another effect of the K\(^+\) Ringer. The high K\(^+\) concentration in the serosal bath resulted in a depolarization of the apical membrane, which in turn activated the Cl\(^-\) conductance (163). In retrospect it is not surprising that Voute and Meyer (331) found that, in short-circuited preparations exposed to Na\(^+\) Ringer on the inside, the much smaller Cl\(^-\) currents were poorly correlated with the number of MR cells.

In toad skin exposed to NaCl Ringer on the inside the Cl\(^-\) currents were activated by clamping the transepithelial potential to \(-100\ \text{mV}\) (338). The Cl\(^-\) currents activated in this way also exhibited a large variation among different preparations, a variation that was further increased by keeping the toads in distilled water before isolation of the skin. With the use of Rudneff’s silver-staining technique (266) for estimating the density of MR cells (\(D_{\text{MR}}\)), the following linear relationship was found between the fully activated Cl\(^-\) current (\(I_{\text{Cl}}\)) and \(D_{\text{MR}}\) (Fig. 8A): 

\[
I_{\text{Cl}} = -2.6 \times 10^{-3} D_{\text{MR}} + 11.6 \ \mu\text{A/cm}^2 (r = -0.96).
\]

Thus these Cl\(^-\)-transporting cells contain membranes ranking among the most conductive membranes in nature.

**B. Current Density Profiles Above Epithelium**

The current density profile above the isolated voltage-clamped epithelium has been studied by moving a vibrating voltage-sensing microelectrode at constant height above the external surface. With this method sites of high conductance are revealed by the voltage drop at the tip of the electrode between the two extreme points of its excursion in the extracellular bath (135). With the known resistivity of the solution and using the simplifying assumption that the electric field is uniform over the distance covered by the electrode tip, a rough estimate of the current density is obtained. In a study using the skin of *R. pipiens* (86), it was not possible to localize peaks of current density to MR cells unless the animals were salt adapted. This treatment reduced the MR cell density by \(-50\%\), whereby the spatial resolution of the method was improved. In such preparations a peak of current density was recorded above 15 of 30 MR cells with a mean value of \(-1\ \text{nA/cell at } -100\ \text{mV}\) (see Fig. 8B). Also some gland ducts were found to be extremely conductive, but they were readily distinguished from the MR cells due to their larger size and the morphology of the cells lining the opening on the surface. In another series of experiments using epithelia of tap water-adapted animals the volume of single MR cells...
was estimated by video-enhanced microscopy. About 50% of MR cells examined underwent a reversible volume increase in response to transepithelial voltage clamping to −150 mV. The voltage-induced cell swelling was dependent on the presence of Cl− in the apical bath only (86). Taken together the results of this study show that MR cells are sites of a large Cl−-specific conductance. However, it was also indicated that not all MR cells possess a conductive Cl− permeability. Katz and Scheffey (140) arrived at a similar conclusion in a study using the vibrating-probe technique for localizing conductance peaks above the isolated epithelium of the skin of salt-adapted B. viridis. Their results indicated that after voltage activation of the transepithelial Cl− conductance, current density peaks above MR cells build up with a time course similar to that of the macroscopic Cl− current of the preparation.

C. Cell Volume Studies

Major information on membrane permeabilities of the MR cells has been obtained by cell volume studies using the optical-sectioning video-imaging technique of Spring and Hope (285). Individual MR cells of the isolated epithelium are visualized with a video system coupled to an inverted microscope equipped with Nomarski optics. Video images containing an optical section of the cell are stored on a video disk for later planimetric analysis. The volume of the MR cell is calculated from estimated cross-sectional areas and the distance between focal planes. As the time-consuming planimetric analysis is performed after the experiment, it is the scanning procedure of 1.5 s (including storage of video frames) that determines the time resolution of the method.

I. Sodium conductive pathways

The addition of 3 mM ouabain to the serosal bath produced a large reversible volume increase that was fully prevented if 50 μM amiloride was present in the outside bathing solution (176; Fig. 9). These results show that the serosal membrane of the MR cell contains a Na+−K+ pump and that the leakage pathway for Na+ entering the cell along its electrochemical potential gradient is located in the apical membrane only. The low concentration of amiloride effectively inhibiting the ouabain response indicates that the apical Na+ permeability is governed by channels similar to the apical Na+ channels of the principal cells.

With a Na+−K+ pump in the serosal membrane and a passive Na+ entry pathway in the apical membrane, it follows that the MR cells contribute to the active uptake of Na+. The steady-state active Na+ flux \( J_{Na}^{active} \), prevailing before the blocking of the pump, was estimated from the initial rate of ouabain-induced volume expansion \( ([\Delta V/\Delta t])_o \)

\[
J_{Na}^{active} = 3C_Na^\circ [\Delta V/\Delta t]_o
\]

where \( C_Na^\circ \) is the Na+ concentration of the external (isosmotic) NaCl solution. Equation 7 assumes that the initial rate of volume increase is determined by the rate at which Na+ enters across the apical membrane while K+ leaves the cell across the serosal membrane, that the Na+/K+ stoichiometry of the pump is 3:2, and that initially the intracellular potential is not changed significantly. The latter assumption is realistic due to the large membrane conductances of the MR cell that effectively reduce the contribution of the rheogenic Na+−K+ pump to the membrane potential. For six MR cells analyzed the mean value was \( J_{Na}^{active} = 0.10 \pm 0.01 \) fmol/s, with an associated short-circuit current of 10 pA/MR cell. This value, corresponding to a specific membrane Na+ current of 80–90 μA/cm², indicates a larger density of Na+ channels in the apical membrane of MR cells compared with that of principal cells generating active Na+ currents of 20–25 μA/cm².
2. Chloride conductive pathways

The ouabain-induced volume expansion was eliminated in the absence of Cl⁻ in the bathing solutions. Unilateral Cl⁻ substitutions with gluconate did not prevent ouabain-induced cell swelling. Thus a Cl⁻-specific permeability is present in both membranes (176). Under short-circuit conditions, MR cell volume decreased by ∼14% in response to bilateral exposure to isosmotic Cl⁻-free solutions (gluconate substitution). If it is assumed that Cl⁻ leaves the cells together with monovalent ions (Na⁺ and K⁺), the initial steady-state Cl⁻ concentration could be estimated from

\[ C_{\text{Cl}}^0 - C_\text{Cl}(V_1 - V_2)/[2(V_1 - x'V_2)] \]  

(8)

where \( C_\text{Cl}^0 \) is the concentration of an isosmotic NaCl solution (115 mM); \( V_1 \) and \( V_2 \) are the control and the Cl⁻-free cell volumes, respectively; and \( x' \) is the fraction of the osmotically inactive volume of the Cl⁻-depleted cell [estimated to be 0.21 (176)]. The value thus obtained, \( C_{\text{Cl}}^0 = 19.8 \pm 1.7 \text{ mM} \), compares well with Cl⁻ concentrations of 14 mM [frog skin (261)] and 25 mM [toad skin (257)] measured with the electron-microscope technique. Elimination of the apical Na⁺ conductance (amiloride or Na⁺-free outer solution) resulted in a volume loss similar to the Cl⁻ space of the cell, indicating that in the short-circuited skin of \textit{R. marinus} the intracellular Cl⁻ concentration is in thermodynamic equilibrium with the Cl⁻ concentration of the bathing solutions. Thus in short-circuited preparations the intracellular potential should be approximately -45 mV, with the cellular K⁺ concentration being far above its equilibrium concentration.

As illustrated in Figure 10A, voltage-induced Cl⁻ conductance activation results in an increase in MR cell volume. The time course of the reversible volume change followed that of the current, and both processes were prevented when the Cl⁻ of the outside bath was replaced with gluconate (Fig. 10B). Over the range of transepithelial potentials at which the steady-state Cl⁻ conductance increases monotonically (see Fig. 4B), the MR cell volume also increases monotonically (Fig. 10C). It was concluded that Cl⁻ conductance activation is associated with the uptake of Cl⁻ across the apical membrane of the MR cells together with K⁺ taken up from the serosal solution (amiloride was present in the outside bath), thereby fulfilling the requirement that the voltage-activated Cl⁻ conductance be localized in the apical membrane of the MR cells. The significance of the serosal membrane K⁺ conductance for the time course of voltage-induced volume expansion in amiloride-treated preparations was demonstrated by showing that the time constant of the volume increase was dramatically increased if the K⁺ concentration of the serosal bath was decreased to 0.5 mM.

Volume expansion per se (bilateral exposure to a hypotonic solution) did not activate the Cl⁻ conduc-

tance, nor was the activated Cl⁻ conductance inactivated by osmotically shrinking the MR cells (176). These findings provide strong evidence that Cl⁻ conductance changes of the apical membrane of MR cells are not controlled by cell volume changes but are triggered by changes of apical membrane potential.

3. Water permeability and volume response to osmotic perturbations

Reduction of the osmolality of the outside solution produced a significant MR cell volume expansion, indicating that the apical membrane of the MR cell possesses a significant water permeability (286). Unless the preparation is stimulated with antidiuretic hormone, the apical membrane of the principal cells is virtually water impermeable (211, 313). These findings suggest that water uptake in unstimulated amphibian skin from diluted outside bathing solution takes place via MR cells of the epithelium.

The osmotic behavior of the MR cells has been investigated by analyzing the relationship between steady-state volume and external osmotic concentration (176). The MR cells were Cl⁻-depleted by apical exposure to amiloride under short-circuit conditions. With bilaterally imposed changes of osmotic pressure the cell volume was well described by the mathematical expression for an osmometer

\[ V/V_0 = [(V_0 - x)/V_0](II_0/II - 1) + 1 \]  

(9)

where \( V_0 \) is the cell volume under isomotic conditions, II₀ is the osmotic pressure of the outside and inside bathing solutions, \( V \) and II are the new values after osmotic perturbations, and \( x \) is the osmotically inactive volume of the cell. Therefore, within a time frame of 10–30 min, Cl⁻-free MR cells do not exhibit regulatory cell volume adjustments.

Mitochondria-rich cells that were not Cl⁻-depleted before osmotic perturbations are capable of volume regulation in a hypotonic serosal bathing solution (286). In response to a 50% dilution of the serosal solution with distilled water the initial MR cell volume increased was followed by a return of the volume to its control value (Fig. 11). With subsequent exposure of the serosal side to NaCl Ringer the cells shrank below their control volume, indicating that the regulatory volume decrease is associated with loss of osmotically active molecules from the cell. In agreement with what has been found for other cells (124, 317), one might speculate that the volume regulatory decrease is the result of cellular KCl loss, here governed, predominantly, by activation of a K⁺ conductance in the serosal membrane. This hypothesis is in agreement with the absence of regulatory volume decrease in Cl⁻-free cells. Clearly more experiments are needed for evaluating the mechanism(s) of regulatory volume responses of MR cells.
January 1991

MITOCHONDRIA-RICH CELLS OF HIGH-RESISTANCE EPITHELIA

D. Studies With Electron-Microprobe Technique

Information on exchangeability of intracellular electrolyte pools has also been obtained by electron-microprobe analysis of MR cells. An X-ray energy spectrum is obtained from areas of 1 μm² of freeze-dried cryosections. Element-specific peaks are calibrated using an albumin Ringer placed on top of the epithelium (51, 259).

The Na⁺ and K⁺ concentrations of the MR cell are similar to those of the principal cells of the epithelium: \( C_{Na} = 11 \text{ mmol/kg wet mass} \), and \( C_{K} = 111 \text{ mmol/kg wet mass} \). The Cl⁻ concentration is significantly smaller than that of principal cells, 12 \( [R. \text{ temporaria, R. esculenta (261)}] \) or 20 mmol/kg wet mass \( [B. \text{ viridis (257)}] \). A more elaborated study indicated a large scatter of \( C_{Cl} \) among MR cells, which led to the suggestion that the transport properties of MR cells in amphibian skin differ, even among cells in the same preparation (49). With ouabain present in the serosal bath, \( C_{Na} \) increased and \( C_{K} \) decreased, but these effects were not as pronounced as in principal cells. The ouabain-induced cation distributions were reduced in preparations exposed to a Na⁺-free outside solution, but they were little influenced by amiloride (260). This early study was the first indicating that ouabain-sensitive Na⁺-K⁺ pumps are located in the serosal membrane of MR cells and that Na⁺ is being taken up across their apical membrane. However, the amiloride sensitivity of the Na⁺ entry pathway, indicated in a subsequent study (176), was not revealed. The basis for these discordant effects of amiloride is not yet clear. Using Br⁻ as a tracer for Cl⁻, X-ray microanalysis has been used also for elucidating pathways for transepithelial Cl⁻ fluxes (49). In short-circuited preparations MR cells took up Br⁻ from either side of the epithelium. Principal cells exchanged Br⁻ across the serosal side only. Voltage-induced activation of the transepithelial Cl⁻ conductance resulted in significantly larger Br⁻ concentrations in MR cells, and it was shown that Br⁻ was taken up from the outside solution. Also these experiments revealed a large scatter of steady-state halide concentrations in MR cells. In MR cells with large uptake of Br⁻ under voltage-activated conditions, the sum of Br⁻ and Cl⁻ concentrations was higher than the Cl⁻ concentration of MR cells in short-circuited preparations. In such cells the Na⁺ concentration was increased, whereas the K⁺ concentration...
and cellular dry mass content were decreased, indicating MR cell volume expansion. In voltage-activated preparations also the principal cells contained Br⁻ when exposed to NaBr Ringer on the outside. Because of the large transepithelial inward Br⁻ fluxes ($I_{Br} \sim -400 \mu A/cm^2$), it was suggested that the principal cells took up Br⁻ from the lateral intercellular spaces. It was concluded that MR cells, making up a heterogeneous cell population, might constitute the cellular locus for transepithelial halide fluxes with no contribution from principal cells (49).

**E. Anion Specificity and Concentration Dependence of Anion Fluxes**

Early studies indicated that the conductive Cl⁻ permeability of frog skin is virtually eliminated if Cl⁻ is removed from the epithelial side (142, 147, 201, 232). In short-circuited preparations, activation of the anion conductance was brought about by apical exposure to Cl⁻ or Br⁻, whereas I⁻, SCN⁻, gluconate, and SO₄²⁻ were without effect. Once activated, however, the conductive anion pathway exhibited a poor anion selectivity according to SCN⁻:Br⁻:Cl⁻:I⁻ = 1.7:1.3:1:0.8. Thus regulation occurs at a Cl⁻ (and Br⁻) ion-specific site remote from a poor anion-selective translocation site of the channel (157). In the absence of external Cl⁻, the channels remain closed even if the transepithelial potential is clamped to a value at which, in the presence of external Cl⁻, they would have been fully activated (109, 163). The voltage-activated anion conductance exhibited the following sequence for ion translocations, Br⁻:Cl⁻:I⁻:SO₄²⁻ = 1.3:1:0.7:0.035 (109, 175). The MR cells seem to constitute the major pathway for sulfate, which behaves as a monovalent anion species (154, 175).

**F. Adenosine 3',5'-Cyclic Monophosphate Dependence of Chloride Conductance**

Besides being controlled by membrane potential and external Cl⁻ concentration, the apical Cl⁻ permeability of the MR cells also seems controlled by an adenosine 3',5'-cyclic monophosphate (cAMP)-dependent pathway (38, 139, 141, 158, 169, 217). The biochemical mechanism of this control is not known. The following is a discussion of how cAMP affects the conductance-voltage relationship of the epithelium.

As mentioned in section IIIA1, the threshold for voltage activation of the Cl⁻ conductance varies among preparations of different species (e.g., see Fig. 4, A and B). A study with frog skin (R. esculenta) revealed that the Cl⁻ currents of some preparations exhibited trivial voltage dependence. Addition of a phosphodiesterase inhibitor (theophylline) to such preparations activated the voltage-sensitive Cl⁻ conductance (158). Adaptation of the euryhaline green toad (B. viridis) to solutions with >100 mM NaCl concentration resulted in a statistically significant reduction of the number of MR cells and a shift of the Cl⁻ conductance-voltage curve of the skin to the left along the voltage axis. As a result, activation of the Cl⁻ permeability would not occur at physiological potentials. Application of the phosphodiesterase...
inhibitor 3-isobutyl-1-methylxanthine to skins of salt-adapted toads shifted the voltage threshold for Cl⁻ conductance activation back into the physiological region of transepithelial potentials (139). The above results indicate that the position of the Cl⁻ conductance-voltage curve along the voltage axis is modulated by the cAMP concentration of the MR cells. For the testing of this hypothesis, 500 μM cAMP was added to the serosal solution of isolated epithelium of toad skin. The apical sodium channels were closed with amiloride. The relations depicted in Figure 12 show that CAMP treatment indeed results in a rightward displacement of the conductance-voltage curve without affecting the fully activated conductance.

V. PARACELLULAR ION PERMEABILITIES

In the first transport model of frog skin it was tentatively assumed that Cl⁻ flows through the Na⁺-transporting cells (147). Later the "Cl⁻ shunt" was associated with the paracellular path (320). As discussed in sections III C2 and IV, recent studies based on different experimental approaches have provided conclusive evidence that a significant component of the passive Cl⁻ current flows through the MR cells and that the apical membrane of the principal cells is Cl⁻ impermeable. This section discusses the significance of the junctional membranes for ion transport across the epithelium.

We have no reason to believe that the junctional permeabilities are fixed and independent of the osmotic conditions to which the animal is adapted. In experiments with frogs, Krogh (162) noted that animals kept in running distilled water at first experienced a fairly large Cl⁻ loss via the skin. Later, however, the loss became negligibly small. We now know that this treatment results in a significant increase in the number of MR cells (62a, 132, 139, 338). Krogh's observations indicate, therefore, that the junctional permeability may be reduced together with an increased capacity for active uptake of Cl⁻ via MR cells (the mechanism of active Cl⁻ transport by these cells is discussed in sect. VII). The passive ion permeabilities increase dramatically during molting (137, 164, 236). The shunt path of molting skins has the property of a free diffusion leak for Na⁺ and Cl⁻. A nonspecific increase in junctional permeability is seen in skins exposed to a hypertonic solution on the outside (67, 318, 320). Osmotically induced permeability changes are fully reversible, but their physiological significance in amphibian skin is, as yet, unknown. In other epithelia, osmotic sensitivity of tight junctions seems to be of significance in controlling transepithelial solute flows (45, 251). Estimates of junctional permeabilities given below are taken from studies of, supposedly, nonmolting skins that were exposed to Ringer, or diluted Ringer, on the outside.

As the apical Cl⁻ conductance of the MR cells is shut and exchange diffusion is eliminated in the absence of external Cl⁻, the Cl⁻ efflux in preparations exposed on the outside to a Cl⁻-free solution should be governed by paracellular pathways. Effluxes in short-circuited preparations (J'<sub>Cl⁻</sub>) exposed to a Cl⁻-free external solution are given in Table 3 together with their associated rate coefficients calculated from k<sub>Cl⁻</sub> = J'<sub>Cl⁻</sub>/C<sub>Cl⁻</sub> where C<sub>Cl⁻</sub> is the Cl⁻ concentration of the serosal bath. It can be concluded that the junctional Cl⁻ permeability (P<sub>Cl⁻</sub><sup>m</sup>) of toad skin is ≤10⁻⁶ cm/s. In the isolated frog skin, P<sub>Cl⁻</sub><sup>m</sup> may be somewhat larger. The values of Table 3, calculated from recent studies, should be compared with data in the first study of the shunt in frog skin (146). In this classic study, the resistance of the shunt was estimated

<table>
<thead>
<tr>
<th>J'&lt;sub&gt;Cl⁻&lt;/sub&gt;</th>
<th>pmol·cm⁻²·s⁻¹</th>
<th>n</th>
<th>k&lt;sub&gt;Cl⁻&lt;/sub&gt;</th>
<th>cm/s</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 ± 0.2</td>
<td>8</td>
<td>0.97 x 10⁻⁸</td>
<td>R. bufon</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 ± 5</td>
<td>6</td>
<td>22 x 10⁻⁸</td>
<td>R. temporaria</td>
<td>155</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28 ± 5</td>
<td>16</td>
<td>24 x 10⁻⁸</td>
<td>L. ocellatus</td>
<td>252</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 ± 3</td>
<td>8</td>
<td>5 x 10⁻⁸</td>
<td>R. pipiens</td>
<td>14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of preparations. Cl⁻ efflux (J'<sub>Cl⁻</sub>) and rate coefficient (k<sub>Cl⁻</sub>) for short-circuited amphibian skin exposed on the outside to chloride-free Ringer.
from unidirectional influxes and effluxes of Cl\(^{-}\) in skins exposed to NaCl Ringer on the inside and 0.1 Ringer on the outside. Already this study revealed the large variation of the passive Cl\(^{-}\) conductance among different preparations, a variation we now, predominantly, ascribe to a variation in the number of Cl\(^{-}\)-conductive MR cells. Accordingly, in so-called “tight skins” the junctional membrane permeability contributes relatively a lot to the overall Cl\(^{-}\) conductance of the skin. The Cl\(^{-}\) shunt resistance (R\(_{Cl}\)) in such skins was \(\sim 74,000 \Omega \cdot \text{cm}^2 (V \approx -60 \text{ mV})\), indicating P\(_{Cl}\) < 6 \(\times 10^{-8}\) cm/s.

Two methods can be applied for estimating the Na\(^{+}\) permeability of the junctional membranes. The first relies on the assumption that the transcellular Na\(^{+}\) efflux is impeded by the serosal membrane and that this, supposedly small, component can be eliminated by inhibiting the apical Na\(^{+}\) permeability with amiloride. The second method is based on the separation of paracellular from cellular fluxes by pre-steady-state flux-ratio analysis (296). Measured Na\(^{+}\) effluxes and calculated rate coefficients are listed in Table 4. In some studies the addition of amiloride had virtually no effect on Na\(^{+}\) efflux (165, 143). In other studies the addition of amiloride produced significant effects: a small stimulation of Na\(^{+}\) efflux (165, 143). In other studies the addition of amiloride had virtually no effect on Na\(^{+}\) efflux (165, 143). For reference and discussion, the rate coefficients are listed in Table 4. In some studies the addition of amiloride had virtually no effect on Na\(^{+}\) efflux (165, 143).

VI. MATHEMATICAL MODELS OF EPITHELIUM

Over a period of 40 years frog skin has served as an experimental model for studies of NaCl transport in high-resistance epithelia. Today much information is available on membrane potentials, intracellular ion concentrations, cell volumes, tracer fluxes, and major membrane currents, including their time-dependent behavior caused by perturbing individual ionic pathways. It is within the scope of mathematical models, which integrate electrophysiological descriptions of ionic flows through passive and active pathways, to make predictions for experimental testing and to reveal relationships that are not being intuitively comprehended. One of the reasons for us to construct a mathematical model of amphibian skin epithelium was to analyze, on the basis of a conceptually clear approach, the effects of intracellular ion distributions and changes in gating variables on the electrical behavior of the epithelium.

With various purposes, execution of this type of analysis has been performed on models of polarized cells of uniform transport properties of the Koefoed-Johnsen-Ussing type separated by junctional membranes. The first models were restricted by steady-state criteria applied to cells of fixed volume (196, 197) or allowing for cell volume changes associated with ionic accumulations and/or depletions (170). Shortly after that, Lew et al. (185) published the first treatment of the non-steady-state behavior. Subsequent papers combined electrophysiological descriptions of the ionic pathways with a voltage-gated permeability (171, 172). The latter type of model was extended by including an arbitrary number of MR cells in parallel with a large principal cell compartment containing a cotransport system in the serosal membrane (173). Like the first treatment (185), all models deal with transient states as a sequence of quasi-stationary states on a time scale much longer than the duration of capacitive currents, which are ignored.

A. Description of Equations

The electrophysiological descriptions of ionic pathways, including steady-state and non-steady-state crite-
ria, are essentially similar in all models published. The model discussed here (173) is of the type shown in Figure 2H, top, which assumes transepithelial ion flows through 1) a major Na⁺-transporting compartment representing the functional syncytium of principal cells, 2) MR cells of specified density and apical area, and 3) a single barrier representing the junctional membranes. The inner and outer compartments are assumed infinitely large, and individual compartments are of uniform composition.

Passive conductors are mathematically described by constant field equations, which for the ionic current, \( I_p \) with membrane permeability, \( P_p \) read

\[
I_j = -P_j \frac{F^2(\psi' - \psi^o)/(RT)}{1 - \exp[z_jF(\psi' - \psi^o)/(RT)]} \times \{C_i - C_j \exp[z_jF(\psi' - \psi^o)/(RT)]\}
\]

where \( j \) is Na⁺, K⁺, or Cl⁻ and \( s \) is the valence. With membrane potentials defined as the potential of the left-side compartment (\( \psi' \)) minus that of the right-side compartment (\( \psi^o \)) (see Fig. 2B), inward currents become positive. Equation 12 provides a good description of the apical sodium current with the apical Na⁺ permeability governed by the following formulation (95)

\[
P_{Na}^{\infty} = P_{Na}^{\infty,max} K_{Na}^o / (C_{Na}^o + K_{Na}^o)
\]

where \( K_{Na}^o \) is an empirical constant, and \( P_{Na}^{\infty,max} \) is the maximum sodium permeability (at zero external sodium concentration). All other permeabilities except the chloride permeability of the apical membrane of the MR cell (\( P_{Cl}^{MK} \)) are assumed to be constant (i.e., independent of voltage and concentration). The gating mechanism controlling \( P_{Cl}^{MK} \) is of a simple Hodgkin-Huxley type (123) defined by the following model for chloride channels

\[
\begin{align*}
\frac{\alpha}{1-s} & \quad \text{channels closed} \\
\frac{\beta}{s} & \quad \text{channels open}
\end{align*}
\]

where \( \alpha \) and \( \beta \) are the forward and backward rate coefficients that are instantaneous functions of the apical membrane potential, and \( s \) is the gating variable (1 ≤ \( s \) ≤ 0) with the Cl⁻ permeability at any given membrane potential and time defined by

\[
P_{Cl}^{MK} = P_{Cl}^{MK,max} g^o
\]

and

\[
\frac{ds}{dt} = \alpha (1-s) - \beta s
\]

\[
\alpha = \frac{V_{Na}^{MK} - \psi^o}{\exp(V_{Na}^{MK} - \alpha \psi^o)/\alpha} - 1
\]

\[
\beta = \frac{1}{\exp(-V_{Na}^{MK} - \alpha \psi^o)/\alpha} + 1
\]

where \( V_{Na}^{MK} \) is the apical membrane potential of the MR cell. Numerical values of the constants \( a \) and \( b \) were chosen for achieving the following empirical features of the macroscopic kinetics: 1) voltage-gated Cl⁻ currents in the physiological range of potentials (they are little activated at short-circuit conditions and often fully activated at \( V \) between -50 and -70 mV); 2) a relatively steep conductance-voltage relationship; \( \beta \) current activations with time courses of ~10–100 s; and \( \beta \) by constructing the \( \beta \)-function with the finite asymptotic value, \( b_0 \), we were able to model the required steep \( G_{Cl}-V \) relationship and, at the same time, obtain the observed fairly large time constant of current inactivations (e.g., see Fig. 6). The following set of values satisfied the above requirements: \( a_1 = 85 \text{ mV}, a_2 = 12 \text{ mV}, a_3 = 0.007 \text{ s}^{-1}, b_1 = 47 \text{ mV}, b_2 = 6 \text{ mV}, \) and \( b_3 = 0.03 \text{ s}^{-1} \).

The Na⁺-K⁺ pump in frog skin is rheogenic with a probable stoichiometry of Na⁺:K⁺ = 3:2 (1, 97, 168, 237, 238, 240). The pump fluxes (superscript P) are represented by the Garay-Garrahan formulation (96), assuming three noninteracting binding sites of identical Na⁺ affinity

\[
J_{Na}^P = J_{Na}^{P,max}[C_{Na} / (C_{Na} + K_{Na}^P)]^p, J_{K}^P = -2J_{Na}^P / 3
\]

where \( J_{Na}^P \) and \( J_{K}^P \) are the Na⁺ and K⁺ pump fluxes. Lew et al. (185) also assumed that serosal K⁺ activates the pump by a first-order binding process, which is preferred if \( C_{Na}^o \) is varied in the computations.

The fluxes carried by the cotransport system (superscript C) of the principal cells are calculated by

\[
J_{Na}^C = iK[C_{Na}^o C_{K}^C / (C_{Na}^o + K_{Na}^C)]^p - C_{Na}^o C_{K}^C (C_{Cl}^C)^p]
\]

where \( i = 1 \) for \( j = \text{Na}^+ \) and \( K^+ \), and \( i = 2 \) for \( j = \text{Cl}^- \). Cell volume (V) adjustments are allowed for by assuming the existence of fixed amounts (Q_\( x \) with mean valency \( z_x \) of impermeant intracellular anion \( x \))
with a concentration $C_x^{c}$ governed by the assumption of isosmolarity in the serosal and cellular compartments

$$C_{Na}^{i} + (2\sigma_{KCl} - 1)C_{K}^{i} - C_{Cl}^{i} = C_{Na}^{c} + C_{K}^{c} + (2\sigma_{KCl} - 1)C_{Cl}^{c} + C_{x}^{c}$$

where $\sigma_{KCl}$ is the KCl reflection coefficient of the serosal membrane, taking into account that both of these ions pass freely through the membrane. Thus in these computations we assume $\sigma_{KCl}$ is different from unity (arbitrarily, $\sigma_{KCl} = 0.95$), noting that results computed with $\sigma_{KCl} = 0.95$ are not significantly different from those obtained by setting $\sigma_{KCl} = 1$.

With $Q_{j}^{c}$ denoting the cellular pool of ion species $j$ and $J_{j}^{a}$ and $J_{j}^{s}$ being total fluxes of $j$ across the apical and serosal membranes, respectively, mass conservations are expressed by the following continuity equations

$$\frac{dQ_{j}^{c}}{dt} = J_{j}^{a} - J_{j}^{s}$$

$$C_{j}^{c} = Q_{j}^{c}/V$$

with the left-hand side of Equation 24 being zero if steady-state solutions are used. At all times during the integration the cells are in osmotic equilibrium with the internal compartment.

Electroneutrality in cellular compartments demands

$$C_{Na}^{i} + C_{K}^{i} - C_{Cl}^{i} + z_{j}C_{x}^{c} = 0$$

Finally, with three pathways available for transepithelial ion flows, the transepithelial net flux density of $j$, $J_{j}$, is

$$J_{j} = n_{A}J_{j}^{MR} + (1 - n_{A})J_{j}^{PE} + J_{j}^{jM}$$

where superscripts denote the MR cell (MR), principal cell (PC), and junctional membrane (jm) pathways; $n_{A}$ is the surface area of the single MR cell, and $n$ is the number of MR cells per unit area of the whole epithelial surface.

Although the above set of equations constitutes a well-posed mathematical problem, the description also includes equations for calculating steady-state transepithelial unidirectional isotope fluxes [on the basis on multicompartment theory (321)] and ionic conductances of the membranes [on the basis of electrodiffusion theory (295)]. The domain of the model is limited. It is emphasized that the model is not, as yet, intended for reproducing certain types of experiments. These include osmotic perturbations of the bathing solutions evoking regulatory cell volume responses. Nor are other secondary permeability changes being accounted for, as, for example, the pH-dependent down- or upregulation of the apical Na$^{+}$ and the serosal K$^{+}$ pump, described in an elegant study by Harvey et al. (115). These regulatory processes seem to occur, for example, in response to changes in cellular Na$^{+}$ concentration. Because the Na$^{+}$-K$^{+}$ pump is described as an irreversible device with no ATP consumption, analyses of reversal potentials of transepithelial active Na$^{+}$ currents cannot be carried out. It is also emphasized that the MR cells of the model are not yet performing what is considered to be their main task when the frog is in the pond, i.e., active uptake of Cl$^{-}$. Likely extensions meeting this requirement presuppose incorporation of an apical ATP-dependent H$^{+}$ transport system in parallel with an Cl$^{-}$-HCO$_{3}^{-}$ exchanger, as shown in Figure 2B. For the present analyses, this deficiency of the model is not of significant importance. This is because we are investigating electrical behavior of the epithelium under conditions where the Cl$^{-}$ conductive properties are prevailing and proton fluxes are not being contained, at any significant level, in apical membrane currents.

### B. Short- and Open-Circuit Conditions

The independent variables are listed in Table 5. Procedures for estimating their physical values have been discussed (171, 173, 185). Briefly, with the short-circuited epithelium taken as the reference state the independent variables are selected so that the model faithfully reproduces experimentally measured intracellular ion concentrations and cell water volumes of the two cell types, intracellular potentials of the principal cell compartment and the MR cell, and principal cell components of the short-circuit current. The fully activated apical Cl$^{-}$ permeability of the MR cells is provided by the estimated Cl$^{-}$ current density of single MR cells at $-100$ mV (338). It is appropriate to mention here that results given by the model have a robust character. Instabilities have never been disclosed in the neighborhood of physical solutions. Furthermore, as long as the broad features of the two-membrane theory (see Fig. 2A) are maintained, the qualitative behavior of the model is not very sensitive to such details as the exact values contained in the input list. With the above requirement of reproducing a specified set of experimental quantities (intracellular concentrations, voltages, currents, cell volumes), the value of each independent variable is limited by a narrow range of possibilities. Our step-by-step procedure of finding these input quantities of the reference state (listed in Table 5) differs from that employed by Lew et al. (185). They developed a program for computing, in one go, the reference state of their model by using as input the selected set of observable quantities.

Model values computed for short-circuit ($V = 0$) and open-circuit ($I = 0$) conditions are listed in Table 6. The intracellular Cl$^{-}$ concentration of the syncytial compartment is above electrochemical equilibrium due to the combined operation of the Na$^{+}$-K$^{+}$ pump and the
cotransport system in the serosal membrane. In contrast, Cl⁻ is passively distributed in the MR cells. The high degree of stability of the epithelium is well illustrated by the fact that intracellular concentrations and cell volumes are not much influenced by releasing the voltage clamp. The small increase in the Cl⁻ concentration of the principal cell compartment (and the associated cell swelling) is caused by the steeper Na⁺ concentration gradient between cell and the serosal bath. In response to shifting from the short-circuit to the open-circuit state, the transepithelial conductance also increases and FRO of the MR cells is being reduced. This is caused by the depolarization of the apical membrane of the MR cells, which in turn is activating the apical Cl⁻ permeability. In this respect, the model is doing what it was asked to do.

C. Testing Model Predictions

The major purpose of our models was to investigate the kind of assumptions needed for reconstructing current-voltage relations and voltage-clamp currents of the real epithelium. The first question we dealt with was whether pure ion distribution currents in a constant permeability regime account for the nonlinear steady-state current-voltage relations and the evolution of time-dependent states produced by transepithelial voltage clamping. The outcome of this analysis, assuming constant field rectification of passive currents, was that hyperpolarizing voltage clamps indeed may lead to a slow increase in transcellular Cl⁻ conductance governed by time constants of 40 s. Thus this relatively simple model exhibited nonlinear current-voltage curves resembling, to some extent at least, those of the epithelium (172, 174). The strong 1 Cl⁻ rectification was found to be due to changes in intracellular Cl⁻ concentration and apical membrane potential. The time-dependent states, caused by the slow accumulation and/or depletion of intracellular pools of Cl⁻ and K⁺, were governed by the serosal membrane's K⁺ conductance. The large outward chloride currents at V < -50 mV were produced only by setting P_C to such a large value that the transepithelial

---

**Table 5. Independent variables of model epithelium**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Symbol</th>
<th>Unit</th>
<th>MR Cells</th>
<th>Principal Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ion permeability, outer membrane</td>
<td>P_{Na}^N</td>
<td>cm/s</td>
<td>3.6 x 10⁻⁶</td>
<td>8.0 x 10⁻⁷</td>
</tr>
<tr>
<td></td>
<td>P_{Na}^P</td>
<td>cm/s</td>
<td>1.0 x 10⁻⁷</td>
<td>1.0 x 10⁻⁷</td>
</tr>
<tr>
<td></td>
<td>P_{Na}^{max}, P_{Na}</td>
<td>cm/s</td>
<td>1.2 x 10⁻⁸</td>
<td>2.0 x 10⁻⁸</td>
</tr>
<tr>
<td>Ion permeability, inner membrane</td>
<td>P_{K}^P</td>
<td>cm/s</td>
<td>8.0 x 10⁻⁵</td>
<td>2.0 x 10⁻⁵</td>
</tr>
<tr>
<td></td>
<td>P_{K}</td>
<td>cm/s</td>
<td>1.3 x 10⁻⁵</td>
<td>3.0 x 10⁻⁵</td>
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<tr>
<td></td>
<td>P_{K}^{max}</td>
<td>cm/s</td>
<td>2.2 x 10⁻⁹</td>
<td>1.4 x 10⁻⁹</td>
</tr>
<tr>
<td>Ion permeability, junctional membrane</td>
<td>P_{Cl}^J</td>
<td>cm/s</td>
<td>5.0 x 10⁻⁴, 2.5 x 10⁻⁸, 1.0 x 10⁻⁸</td>
<td></td>
</tr>
<tr>
<td>Na⁺ pump constant</td>
<td>f_{Na}</td>
<td>mol·cm⁻²·s⁻¹</td>
<td>1.8 x 10⁻⁹</td>
<td>0.7 x 10⁻⁹</td>
</tr>
<tr>
<td>Cotransport constant</td>
<td>K</td>
<td>cm¹·mol⁻¹·s⁻¹</td>
<td></td>
<td>9.0 x 10⁶</td>
</tr>
<tr>
<td>Reflection coefficient</td>
<td>η_{Cl}</td>
<td>Dimensionless</td>
<td>0.95</td>
<td>0.95</td>
</tr>
<tr>
<td>Nondiffusible anions (valency)</td>
<td>Q_0 (z_i)</td>
<td>mol/cm²</td>
<td>2.2 x 10⁻⁷ (-2)</td>
<td>1.75 x 10⁻⁷ (-2)</td>
</tr>
<tr>
<td>Apical membrane area</td>
<td>A</td>
<td>cm⁻²</td>
<td>1.2 x 10⁻⁷</td>
<td></td>
</tr>
<tr>
<td>MR cell density</td>
<td>n</td>
<td>cells/cm²</td>
<td>6 x 10⁴</td>
<td></td>
</tr>
</tbody>
</table>

Standard concentrations of diffusible ions in external compartments are C_{Na} (=C_{Na}^E) = 112 mM; C_{K} (=C_{K}^E) = 2.4 mM; C_{Cl} (=C_{Cl}^E) = 114.4 mM. Parameters of gating reaction are given in text (Eq. 14-19).

**Table 6. Electrophysiological values given by model at short-circuit and open-circuit conditions**

<table>
<thead>
<tr>
<th></th>
<th>Mitochondria-rich cell compartments*</th>
<th>Principal cell compartment</th>
<th>Mitochondria-rich cell compartments*</th>
<th>Principal cell compartment</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{Na}, mM</td>
<td>12.2</td>
<td>11.9</td>
<td>8.9</td>
<td>8.8</td>
</tr>
<tr>
<td>C_{K}, mM</td>
<td>134.9</td>
<td>129.9</td>
<td>138.1</td>
<td>131.7</td>
</tr>
<tr>
<td>C_{Cl}, mM</td>
<td>19.5</td>
<td>39.6</td>
<td>20.0</td>
<td>44.2</td>
</tr>
<tr>
<td>Volume, nl/cm²</td>
<td>24.8</td>
<td>3,401</td>
<td>24.9</td>
<td>3,605</td>
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<tr>
<td>V, mV</td>
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<td></td>
<td></td>
<td>-24.3</td>
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<tr>
<td>τ⁺, mV</td>
<td>-44.7</td>
<td>-84.4</td>
<td>-29.0</td>
<td>-63.2</td>
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<tr>
<td>I_{Cl}, μA/cm²</td>
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<td>0</td>
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<tr>
<td>G_{j+}, mS/cm²</td>
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<td>0.50</td>
<td>0.62</td>
<td>1.26</td>
</tr>
<tr>
<td>FR_o</td>
<td></td>
<td></td>
<td>0.87</td>
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</tr>
</tbody>
</table>

Model values of intracellular concentrations (C_i), apical membrane potentials (V⁺), volumes transepithelial conductance (G), and fractional resistance of apical membrane (F_{R_o}) under short-circuit conditions (V = 0, I_{SC} is short-circuit current generated by 2 cellular units) and open-circuit conditions (current clamp conditions, I = 0). * 60,000 MR cells/cm². Volume = 413 μm³/MR cell (V = 0 mV). Apical membrane area = 12 μm²/MR cell. Fractional membrane area of MR cells is 0.0072. τ⁺ junctional membrane conductance = 25 μS/cm².
current at \(0 < V < 50\) mV also contained a large Cl\(^-\) component. However, our experiments have established that at \(V > 0\) mV the epithelium conducts vanishingly small Cl\(^-\) currents. Furthermore, transient currents associated with cellular KCl accumulation were found to build up without delay and with time constants that increase with the amplitude of the transepithelial voltage clamp pulse. This latter property, caused by constant field rectification of the serosal membrane K\(^+\) conductance, bears no resemblance to the bell-shaped \(t-V\) relation of the epithelium.

Incorporation of a voltage-gated apical Cl\(^-\) permeability in the MR cells constituted the logical extension of this first tentative description. Before discussing voltage-clamp currents generated by this version of the model, it is important to investigate how accurate the model predicts results obtained by perturbing other membrane reactions in the living tissue. Performing this type of analysis provides useful information concerning the validity of our assumptions regarding the electrophysiological description of individual pathways and the choice of permeability and pump constants.

There are many ways in which such an analysis can be carried out. Most profitable, however, is to choose a few classic laboratory protocols leading to well-known and quantitatively well-described responses of the epithelium, including the time course of its transient states. To begin with, therefore, we discuss model behavior in response to arresting pumps and to perturbing passive currents by blocking the Na\(^+\) channels of apical membranes and by changing their electrical driving forces.

1. Effect of ouabain

The Na\(^+\)-K\(^+\) pumps of the two cellular units create the ion gradients across the membranes on which membrane potentials, cell water volume, and transcellular fluxes of Na\(^+\) and Cl\(^-\) depend. The time course of gradient dissipations and associated changes in fluxes were first analyzed by Lew et al. (185). As our present model incorporates a cotransport system in the serosal membrane of the principal cells, on which their water volume and intracellular Cl\(^-\) concentration depend, it is worth-while taking up this analysis again for investigating whether the powerful predictions of the first description are retained and whether some of its shortcomings are eliminated. Recently, the volume response of the MR cells to pump inhibition has been well characterized (176), so the computed time course of the MR cells volume response is also subject to testing. Ouabain treatment is simulated by an instantaneous reduction of \(J_{\text{pump}}\) by a factor of \(\approx 250\) (Eq. 20), whereby the pump fluxes of Na\(^+\) and K\(^+\) become negligible.

I) PRINCIPAL CELL COMPARTMENT. The short-circuit current exhibits a very slow decrease in response to this treatment (Fig. 13A), as was found in experiments with frog skin (252). This is because the electrochemical potential gradients of Na\(^+\) and K\(^+\) between cells and bathing solutions maintain significant currents through the cells carried by Na\(^+\) across the apical and K\(^+\) across the serosal membranes. The immediate response to pump inhibition is an intracellular depolarization of \(-5.2\) mV, resulting from sudden elimination of the pump current (Fig. 13B). With respect to the response of the short-circuit current and \(V\), there is nothing new to add to the previous model (185) and experimental (230, 231) analyses. It is a novel and interesting result of the present treatment, however, that the cell volume of the principal cell compartment, which does not change much during the first minutes of ouabain exposure, exhibits a slow decrease that lasts 20–30 min before the cell starts swelling inevitably. Such a response, which is somewhat puzzling, was reported in experiments with frog skin (211). Under isosmotic conditions, cell volume changes are governed by the direction of the total net flux of Cl\(^-\), which is the sum of the electrodiffusive flux carried by the small serosal membrane Cl\(^-\) conductance and the flux carried by the cotransporter. From the steady-state concentrations of the three diffusible ions (Table 6), it can be seen (Eq. 21) that the cotransporter carries net fluxes from the serosal bath into the cell before ouabain exposure. Because of the subsequent changes of intracellular Na\(^+\) and K\(^+\) concentrations (Fig. 13C), the equilibrium condition of the cotransporter is approached, and the time marked by an asterisk in Figure 13C the direction of cotransport is reversed, generating a net flux of Cl\(^-\) out of the cell. After \(\approx 20\) min the cotransporter is again operating near equilibrium so that the subsequent trivial net loss of Cl\(^-\) is caused by the passive flux directed from cell to (serosal) bath, which continues until Cl\(^-\) comes in thermodynamic equilibrium. At \(\approx 27\) min the Cl\(^-\) equilibrium potential of the principal cells \((V_{Cl}^\text{eq})\) is equal to \(V_{Cl}^\text{eq} = -33\) mV \((C_{Cl}^\text{eq} = 30\) mM). From then on the principal cell compartment gains Cl\(^-\), and its volume increases (Fig. 13A).

The rate at which these changes occur depends, initially, on the Na\(^+\) and K\(^+\) permeabilities of the apical and serosal membrane, respectively. It is not a point of serious criticism that gradient dissipation in the living tissue may occur on a somewhat longer time scale (e.g., 1 h) than that of computed changes. In amphibian skin the apical Na\(^+\) permeability decreases in response to the slow accumulation of intracellular Na\(^+\) (11, 69, 114, 165), slowing down the rate of further gradient dissipations. As mentioned, this type of reaction is not considered in the model. What is of importance, however, is that the computed ouabain-induced changes of short-circuit current, membrane potential, intracellular ion concentrations, and cell volume are all in good agreement with those observed in experiments with frog skin and that they are predicted to occur, at any rate, with long time constants.

II) MITOCHONDRIA-RICH CELLS. The Na\(^+\) and K\(^+\) pools of the MR cell exchange relatively fast with the external bathing media. After inhibition of the pump fluxes the large passive fluxes of these ions lead to rapid cellular Na\(^+\) accumulation and K\(^+\) depletion. Initially, the electrical driving force acting on these ions changes little (Fig. 13B) so there is a net gain of cations accompa-
nied by cellular gain of Cl⁻. Accordingly, the MR cell volume does not show the same degree of stability as the principal cell compartment (Fig. 13, A and B). The computed rate at which the volume of the MR cell occurs is within the range of measured rates (176; cf. Figs. 13A and 9B).

The intracellular potential is predicted to change little (in the actual computations, by less than −0.1 mV) in response to sudden removal of the pump current. This is because the specific membrane conductance of the MR cell is large. With actual values of membrane permeabilities, ion concentrations, and intracellular potentials, the total membrane conductance (\(G_{\text{cell}}\)) is 46 nS/ MR cell. Before inhibition of the pump, the pump current (\(I_{\text{pump}}\)) was 3.2 pA/MR cell. Under the prevailing transepithelial thermodynamic equilibrium conditions there are no current loops via paracellular or other cellular pathways. Thus the electrogenic contribution of the pump to membrane potential can be estimated by

\[ V_{\text{pump}} = -I_{\text{pump}} / G_{\text{cell}} \]

which gives \(V_{\text{pump}} = 0.07 \text{ mV}\). This result is of significance also for understanding the instantaneous response of the MR cell to sudden closure of apical sodium channels, as is discussed next.
2. Effect of amiloride

Amiloride effects are studied by setting the apical membrane's sodium permeabilities to 10⁻¹¹ cm/s, whereby the apical influxes of this ion become practically zero. Lew et al. (185) gave a fairly complete treatment of the evolution of time-dependent states of the principal cell compartment. Results obtained with the present model, under short-circuit conditions, are not different and are not repeated here. Instead we discuss the new features of the two-cell-type model that were not within the scope of previous treatments. To begin with, however, the electrogenic contribution of the pump to the membrane potential of the principal cell compartment, as revealed by the amiloride response, is discussed.

I) PRINCIPAL CELL COMPARTMENT. Eliminating the apical Na⁺ permeabilities brings the short-circuit current to zero (Fig. 14A), and the principal cell compartment is instantaneously hyperpolarized to -107.3 mV (Fig. 14B). With a K⁺ equilibrium potential of -100.8 mV, the electrogenic contribution of the pump to the membrane potential is at least -6.5 mV. The actual value is better estimated from the pump current (10 μA/cm²) and the total cell membrane conductance at t = 0 but with Na⁺ channels closed (1.08 mS/cm²), giving \( V_{\text{pump}} = -9.3 \) mV, which is more than that obtained (-5.2 mV) with ouabain. The difference of about -4 mV is due to the smaller cell membrane conductance, mainly because the apical Na⁺ conductance has been removed but also because the serosal membrane's K⁺ conductance becomes smaller at large negative membrane potentials (constant field rectification). During the following minutes the Na⁺-K⁺ pump empties the cellular Na⁺ pool, the pump current vanishes, and the intracellular potential approaches the K⁺ equilibrium potential. Due to finite Na⁺ and Cl⁻ conductances in the serosal membrane, the new steady-state intracellular potential, -97.7 mV, is somewhat smaller than \( V_F = -104.5 \) mV. These effects were studied in detail with the microelectrode technique for measuring intracellular potentials (230, 231) as well as K⁺ activities (97) in the principal cell compartment of frog skin. Results obtained in experiments and by model calculations agree well.

II) MITOCHONDRIA-RICH CELLS. Because of the significant apical Na⁺ permeability in the MR cells, the steady-state intracellular potential (-44.7 mV, under short-circuit conditions) is far from the K⁺ equilibrium potential of the serosal membrane. At this potential the apical Cl⁻ permeability is no more than ~12% of its fully activated value and only ~8% of the Cl⁻ conductance of the serosal membrane. Nevertheless, the Cl⁻ conductance of the apical membrane is ~30 times higher than its Na⁺ conductance. Initially, the MR cell potential is not significantly influenced by blocking the apical Na⁺ channels (Fig. 14B). This is because the cell membranes contain such a large Cl⁻ conductance that the cell is "clamped" by the Cl⁻ equilibrium potential \( V_{\text{MR}} = -44.7 \) at \( t = 0 \). This is an important result, because it suggests that the presence of an, even consider-

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**FIG. 14.** Computed response of model epithelium to sudden blockage of apical Na⁺ channels (time zero, arrows), \( V = 0 \) mV. A: \( I_w \) response. B: intracellular potentials of 2 cellular units exhibit different time-dependent responses (\( V_F, V_{\text{mr}} \)). C: Na⁺ and Cl⁻ concentrations and volume of single MR cell. D: apical membrane Cl⁻ permeability (\( P_{\text{Cl,im}} \)) and transepithelial conductance (\( G_t \)).
able, apical Na⁺ permeability in this cell type is not being revealed by amiloride-induced membrane hyperpolarization characterizing classic Na⁺-transporting cells in high-resistance epithelia. The continued operation of the Na⁺-K⁺ pump depletes the intracellular Na⁺ pool. Consequently, the intracellular Cl⁻ pool is also depleted (Fig. 14C). The associated cell volume decrease compares with that measured experimentally (176; see Fig. 9). The agreement between experimental results and model calculations holds with respect to the magnitude of volume change and the time scale.

The slow decrease in $C_{\text{Cl}}^{\text{MR}}$ brings $V_{\text{Cl}}^{\text{MR}}$ toward more negative values, and the cell hyperpolarizes with a similar time course (Fig. 14B). In turn, the apical Cl⁻ permeability is slowly inactivated, which is reflected in a similarly slow decrease in transepithelial conductance (Fig. 14D). An example of an actually recorded amiloride-induced conductance change in frog skin is shown in Figure 15. With respect to its time course, there is some variation (68, 138, 234). However, they all exhibit, in agreement with the model, a “dual” conductance response. This can now be explained in terms of an initial closure of Na⁺ channels and a subsequent slow inactivation of apical Cl⁻ channels of MR cells. With the insight accomplished by model analysis, we also appreciate that the time course of the slow conductance decrease reflects the rate at which the ion pools of the MR cells are depleted by the sodium pump.

Before “amiloride treatment” the Cl⁻ efflux of 53 pmol·cm⁻²·s⁻¹ contains an MR cell component of 52 pmol·cm⁻²·s⁻¹ and a paracellular component of 1 pmol·cm⁻²·s⁻¹. At the new steady state of inactivated apical Cl⁻ channels only the small paracellular component remains. In experiments with frog skin, it has been demonstrated that amiloride indeed eliminates a major component of the transepithelial Cl⁻ fluxes (27, 155). Our treatment provides the explanation for this effect. There is no need for assuming direct amiloride block of Cl⁻ channels.

Also another type of experimental results indicate “coupling” between active Na⁺ flux and passive Cl⁻ flows. In short-circuited frog skin, replacement of external Na⁺ with a nonpermeating cation (e.g., choline, K⁺) produced a significant reduction of the transepithelial Cl⁻ flux measured with $^{36}$Cl (155, 207, 265). Moreover, stimulation of the short-circuit current by application of the Na⁺ channel activator, benzimidazolylguanidine (BIG) produced a significant stimulation of the tracer flux of Cl⁻ (156). The explanation for these results is now fairly obvious. The two situations are other ways of changing the apical Na⁺ conductance to produce cellular hyperpolarization (Na⁺ replacement) or depolarization (BIG), which in turn inactivates and activates, respectively, the apical Cl⁻ permeability of the MR cells through which Cl⁻ is flowing.

3. Current-voltage relations

The steady-state current-voltage curves given by the model are shown in Figure 16A. The very small active outward Cl⁻ current (carried by an inward Cl⁻ flux; see Fig. 3A; $V = 0$ mV) is not included in the model, otherwise it produces the experimental current-voltage relations fairly well. The features that are correctly reproduced are 1) the presence of outward-going rectification, 2) at $V > 0$ mV the Cl⁻ currents are vanishingly small so that the total current contains, for all practical purposes, a single component, that is, the inward Na⁺ current; and 3) at $V < 0$ mV the Cl⁻ currents are significant to the total current, and at large negative transepithelial potentials, $I_{\text{Na}}$ is so small that the passive chloride conductance of (MR cells) governs the total current flow across the epithelium. At first sight, the potential dependence of the steady-state conductance of the model, by showing no saturation, disagrees with that of the epithelium (cf. Fig. 16B with Fig. 4A). Also shown in Figure 16B is the variation of the steady-state apical Cl⁻ permeability of the MR cells with the transepithelial potential difference. In the model the membrane permeability saturates while the transepithelial conductance continues increasing at $V < -50$ mV. This is due to the rectifying property of open channels governed by the constant-field formulation for Cl⁻ movement (Eq. 12). This description also makes the computed $I_{\text{Cl}}$ vs. $V$ curve concave toward the current axis so that, at a large
negative V, the $I_{Cl} - V$ relationship is steeper than the measured linear $I_{Cl} - V$ graph (339). It seems that the equation for electrodiffusive flow of ions through the apical Cl$^-$ channels would require further refinement to take proper account of these features. By giving the required steady-state current-voltage relations over the wide V range of $-100 < V < 50$ mV, the model is reproducing well the electrical properties being most relevant for our purposes.

Confidence in model predictions concerning steady-state dynamics of the apical Cl$^-$ permeability is also gained from comparing relationships of the voltage dependence of computed and experimental efflux of Cl$^-$ ($J_{Cl}^{out}$; Fig. 17, A and B, respectively). Notice stimulation of $J_{Cl}^{out}$ when stepping its driving force down from 0 to, e.g., $-50$ mV. In the model this result is explained by the opening of voltage-gated Cl$^-$ channels in the apical membrane of the MR cells. The I efflux exhibited a similar V dependence (109). Also shown in Figure 17 is the effect of further decreasing V. This removed a major component of $J_{Cl}^{out}$ (and $J_{Cl}^{in}$) in toad skin (24, 56, 109, 339). The model predicts this effect and explains it as the result of decreasing the electrical driving force under conditions of fully activated Cl$^-$ permeability of the MR cells.

Stepping V from zero into the negative region of the current-voltage relation depolarizes the apical membrane of the MR cells and, as discussed in section VI.B, the apical Cl$^-$ permeability increases. The computed cellular Cl$^-$ accumulations and cell volume gains are shown in Figure 18. Within experimental errors, and allowing for the variation in results obtained with different cells, the measured voltage dependence of cell volume (176; see Fig. 10B) and intracellular Cl$^-$ concentration (49) both agree with computed results.

Nevertheless, with respect to these predictions, there is a difficulty. While the model MR cell exhibits a decrease in cellular Na$^+$ concentration (Cl$^-$ is taken up together with K$^+$ derived from the serosal compartment), the Na$^+$ concentration showed a substantial increase in the MR cell of toad skin when estimated by the electron-microprobe technique (49). The reason for the model showing a decreased intracellular Na$^+$ concentration is that the electrical driving force of Na$^+$ entrance across the apical membrane decreases as V is clamped at negative values. For example, at V = $-75$ mV, the steady-state apical membrane potential of the model MR cell is 4.3 mV (cell positive), $C_{Na}^{MR} = 3.0$ mM, and the cell volume is 457 $\mu$m$^3$. If the computed data given in Table 6 is used (V = 0 mV), it is seen that, in response to pulsing V from 0 to $-75$ mV, the model MR cell experi-
at the holding potential, the onset of current activation is delayed in toad skin was not removed by shifting the holding potential toward zero or negative values. It seems incorrect, therefore, assuming that the rate coefficients are independent of the prehistory of the membrane (Eqs. 18 and 19). When activated from a positive holding potential the currents are not increasing monotonically toward their new steady-state value but exhibit a small "overshoot" with maximum at ~60 s. This is because the apical Cl- permeability becomes fully activated before the intracellular concentrations have reached their new steady-state values. Of major significance is a relatively large decrease in ClMR from 43.4 mM (V = 50 mV) to, e.g., 28.9 mM at steady state at V = -75 mV. (The electrical driving force for Cl- exit across the serosal membrane becomes large when V is shifted from 50 to -75 mV.)

Because the rate of cellular Cl- loss is determined by the relatively small cation permeabilities, the "time constant" of intracellular Cl-concentration change is significantly longer than that of Cl- permeability activation. The continued decrease in ClMR after full activation of PCl results in a slow Cl-conductance decrease. To summarize, due to the relatively fast Cl- permeability activation the Cl- current across the apical membrane increases to a maximum value (e.g., 116 μA/cm² at V = -75 mV). Subsequently the apical Cl- conductance decreases, so that the Cl- current at steady state becomes somewhat smaller (~102.3 μA/cm² at V = -75 mV).

Pulsing the transepithelial potential from 50 to -75 mV brings the apical membrane potential from -74.4 mV (cell negative) to 50.3 mV (Fig. 19C). The two rate coefficients instantaneously take up their new values. Because PCl activates, the fractional resistance of the apical membrane starts decreasing, resulting in a slow decrease in the apical cell membrane potential. Accordingly, α decreases again (Eq. 18), and τ increases (Eq. 17).

The general conclusion to be drawn from this example is that there are two reasons that Cl- current activations do not contain the required information on the kinetics of the permeability activation: 1) the time course of the Cl- conductance change is different from that of the permeability change, and 2) the permeability activation is not governed by a single time constant. The latter point is perhaps best illustrated in Figure 20, comparing the τ-V relations calculated at the onset of the voltage pulse (τ0) and at the new steady state (τ*). It is gratifying of course that the type of t-V relation shown in Figure 6B is reproduced by the model. However, detailed quantitative analysis of this type of experimental relation is meaningless. There are some qualitative features of the kinetics of permeability activation that can be investigated by comparing measured and computed voltage-clamp currents. It is a fundamental property of the Hodgkin-Huxley equations that they predict the disappearance of delay in current activation when the membrane potential is brought from a value where the permeability is already partially activated. The model reproduces this feature (171). However, the delay in current activations in toad skin was not removed by shifting the holding potential toward zero or to negative values. It seems incorrect, therefore, assuming that the rate coefficients are independent of the prehistory of the membrane (Eqs. 18 and 19). When V is

FIG. 18. Predicted steady-state volume (left) and intracellular Cl- concentration (right) of MR cell as a function of transepithelial clamping potential. Open circles, apical sodium permeability set at its physiological value. Closed circles, apical sodium channels blocked with amiloride. Computed volume-V relationship compares well with actually measured relationship (cf. Fig. 10B).

enced a net Na+ loss of 3.7 fmol pumped out across the serosal membrane in excess of the Na+ diffusing from bath to cell. In our experiments, ouabain-induced MR cell volume gain was fully prevented by addition of amiloride to the external bath (176; see Fig. 9). On this entirely sound argument, Na+ entrance in the MR cells was modeled as electrodiffusion, with the major permeability being in the apical membrane. Turning now to electron-microprobe data, the ouabain-induced Na+ gain, which was not substantial, could not be prevented by adding amiloride to the apical bath. This observation was taken to indicate, in agreement with the Munich group’s later studies, that another process other than apical electrodiffusion brought Na+ into the MR cell (49, 260).

The conclusion here is that a model incorporating amiloride-sensitive Na+ channels in parallel with a voltage-controlled Cl- permeability in MR cells of the epithelium powerfully predicts a substantial amount of experimental results whether they are being produced by inhibiting cation fluxes of the two cellular units or by transepithelial voltage clamping.

D. Reconstructed Voltage-Clamp Currents

For discussing the main features of the model’s voltage-clamp currents we are using only a single set of independent variables, that is, the one used in the computations presented. It is, of course, a major advantage of mathematical modeling that effects of choosing different sets of variables can be explored. To some extent, this type of analysis has been carried out (171, 173), and, when suitable, the conclusions of significance for the discussion are mentioned.

Voltage-clamp currents of the model are shown in Figure 19A, and in Figure 19H are shown the currents flowing through the MR cells. It can be seen that they resemble those of the epithelium and that the major time-dependent component is the gated current of the MR cells. Because the Cl- conductance was inactivated at the holding potential, the onset of current activation

is sigmoidal as defined by Equation 14. When activated from a positive holding potential the currents are not increasing monotonically toward their new steady-state value but exhibit a small "overshoot" with maximum at ~60 s. This is because the apical Cl- permeability becomes fully activated before the intracellular concentrations have reached their new steady-state values. Of major significance is a relatively large decrease in ClMR from 43.4 mM (V = 50 mV) to, e.g., 28.9 mM at steady state at V = -75 mV. (The electrical driving force for Cl- exit across the serosal membrane becomes large when V is shifted from 50 to -75 mV.) Because the rate of cellular Cl- loss is determined by the relatively small cation permeabilities, the “time constant” of intracellular Cl-concentration change is significantly longer than that of Cl- permeability activation. The continued decrease in ClMR after full activation of PCl results in a slow Cl-conductance decrease. To summarize, due to the relatively fast Cl- permeability activation the Cl- current across the apical membrane increases to a maximum value (e.g., 116 μA/cm² at V = -75 mV). Subsequently the apical Cl- conductance decreases, so that the Cl- current at steady state becomes somewhat smaller (~102.3 μA/cm² at V = -75 mV).
The apical sodium permeability of the syncytial compartment. It is possible then to investigate whether the compartment was increased over the physiological range in range of physiological variation (fixed at 6 $\times$ 10$^{-4}$/cm$^2$).

The MR cell density was, as above, within its controlling the Cl$^-$ permeability activation of MR cells, apical Na$^+$ permeability of the syncytial compartment is returning via MR cells and paracellular restriction, the current flowing through the syncytial equations governed by $I = 0$. With the imposition of this selection to reproduce measured quantities at short-circuit conditions and the fully activated Cl$^-$ current, the model predicts fairly well the observed time-dependent exponential-like time course, but in this respect the range of observations is wide (49, 56, 163, 166, 171, 176, 339; see Fig. 6). There are ways of reproducing such a time course in the model, for example, by choosing smaller values of the inner membrane's permeabilities (173). We were unable, at the same time, to maintain an acceptable representation of the kinetics of current activations. Clearly then, our description of the apical Cl$^-$ channels of the MR cells does not cover all aspects of experimental observations. This is also a suitable point to recall that external Cl$^-$ (or Br$^-$) is needed for the voltage-induced activation to occur (109, 163). In its nonconductive state the transport system may function as an exchange pathway for anions (sect. VIIA). Modeling these features requires further refinement of the equations describing apical transport.

E. Cell Coupling via Extracellular Current Loops

Once permeabilities and pump constants have been selected to reproduce measured quantities at short-circuit conditions and the fully activated Cl$^-$ current, the model predicts fairly well the observed time-dependent states as well as the steady states within a wide range of transepithelial potentials, including the spontaneously developing potential. It is to be expected, therefore, that the model is also revealing essential properties of the epithelium in situations of varying open-circuit potentials, i.e., under transepithelial current-clamp conditions governed by $I = 0$. With the imposition of this restriction, the current flowing through the syncytial compartment returns via MR cells and paracellular junctions. It is possible then to investigate whether the apical Na$^+$ permeability of the syncytial compartment is controlling the Cl$^-$ permeability activation of MR cells (173). The MR cell density was, as above, within its range of physiological variation (fixed at 6 $\times$ 10$^4$/cm$^2$).

The apical sodium permeability of the syncytial compartment was increased over the physiological range in suitable steps from 10$^{-8}$ to 10$^{-6}$ cm/s. Thus the model generated steady-state transepithelial potentials that varied from about $-10$ to $-60$ mV. The associated depolarization of the apical membrane of the MR cells changed the Cl$^-$ permeability from an inactivated to a fully activated state, and the inward net fluxes of Na$^+$ and Cl$^-$ increased about two orders of magnitude, however, with Na$^+$ flowing through the syncytial compartment and Cl$^-$ flowing through the MR cells. In other words, the model predicts that the apical Cl$^-$ permeability of the MR cells is activated according to the active Na$^+$ current generated by the syncytial compartment.

VII. ACTIVE CHLORIDE TRANSPORT

Krogh's studies with frogs (R. esculenta) indicated that Na$^+$ as well as Cl$^-$ is transported through the skin at the expense of metabolic energy and showed that absorption of Cl$^-$ also takes place in the absence of external Na$^+$ (161, 162). These in vivo studies were extended to other species (R. arvalis, R. temporaria, R. esculenta, and B. bufo), and by way of flux-ratio analysis, direct evidence for active Cl$^-$ transport was provided (173a).

The active mechanism has been studied in the in vitro preparation exposed to an external Cl$^-$ concentration of <2 mM (60, 66, 153, 219). In the isolated skin of the South American species, Leptodactylus ocellatus, large active fluxes of Cl$^-$ have also been reported in the presence of NaCl Ringer on the outside (341). Generally, however, in this situation the passive conductance becomes activated (sect. VII E) and as a result the active Cl$^-$ flux vanishes [R. temporaria, R. esculenta (146, 321)] or remains just as a relatively small component of large Cl$^-$ fluxes [B. bufo (24), B. viridis (139), R. pipiens (54), B. arenarium (9)]. Amiloride added to the external bath did not abolish the active Cl$^-$ transport (9, 56, 66, 153). The isolated preparation generates active Cl$^-$ fluxes in the absence of external Na$^+$ (153, 339), confirming the above-mentioned conclusion of in vivo studies, that the active Cl$^-$ influx is carried by a Na$^+$-independent transport system.

A plausible hypothesis for the mechanism of active Cl$^-$ transport (70) is depicted in Figure 2B, bottom. It is assumed that the transport mechanism is in the apical membrane of the MR cells. Explicitly, the model suggests how metabolic energy is used to move chloride against an electrochemical potential gradient. It should be noted that the active flow of Cl$^-$ according to this model is rheogenic, which is in agreement with experimental findings (24, 56, 339, 341).

In the next section it is shown that this model is reconciled with published experimental observations, including those of a recent study (176a) designed for a more direct testing of the model.

A. Chloride-Bicarbonate and Chloride-Chloride Exchange

The idea of coupling between active uptake of chloride and secretion of bicarbonate goes back to Krogh
Kirschner (144a) considered a similar function of the apical membrane of both principal and MR cells. The electrical driving force for passive Na\(^+\) uptake across the apical membrane of the MR cells was also considered in subsequent studies. Recently, Ehrenfeld and Harvey (62a) suggested that the H\(^+\) pump is located in the MR cells and that under open-circuit conditions it is generating an electrical potential gradient.

B. Proton Transport

Ehrenfeld and Garcia Romeu (59) suggested that an apical electrogenic proton pump in the principal cells of frog skin provides the electrical driving force for Na\(^+\) uptake from low external Na\(^+\) concentrations. Such a function of the pump was also considered in subsequent detailed studies of the rheogenicity and energy requirement of proton fluxes generated by isolated frog skin (61, 111). Recently, Ehrenfeld and Harvey (62a) suggested that the H\(^+\) pump is located in the MR cells and that under open-circuit conditions it is generating an electrical driving force for passive Na\(^+\) uptake across the apical membrane of both principal and MR cells. Kirschner (144a) considered a similar function of the proton pump. The basis for this hypothesis is discussed in section VII.C.

In the next section is a general description of the acid-secreting mechanism of amphibian skin, followed by a discussion of another function of the proton pump in MR cells, i.e., to drive chloride against an electrochemical potential gradient by providing the anion exchanger with cellular bicarbonate.

1. Features of proton excretion in amphibian epidermis

It is well established that frog skin has the capacity of acidifying the outside solution whether sodium is taken up from low concentrations of its salt (59, 84, 98, 127, 143, 208, 311) or from Ringer solution (64, 65, 208). Evidence for an active rheogenic proton pump, rather than an obligatory Na\(^+\)-H\(^+\) countertransporter, has been provided by three types of experiments. 1) Proton secretion by frog skin was not significantly affected by substituting external Na\(^+\) with K\(^+\) (208) or Mg\(^{2+}\) (65). 2) In the living frog, proton secretion and Na\(^+\) absorption are coupled with a 1:1 stoichiometry (98). 3) Proton secretion was abolished by oxygenation (61, 64) and by metabolic inhibitors, e.g., dinitrophenol and antimycin (208), indicating dependence on oxidative metabolism. Proton secretion was also blocked by proton pump inhibitors, such as dicyclohexylcarbodiimide (DCCD) and oligomycin (61).

Proton secretion can be reversibly abolished by carbonic anhydrase inhibitors (59, 61, 64, 208), providing indirect evidence for the proton pump being in the carbonic anhydrase-containing MR cells. This assumption is in line with the finding that a freeze-fracture replica of the apical region of MR cells in amphibian epidermis contains rod-shaped particles on their P-face like MR cells of distal renal epithelia (21). It is believed that these intramembrane particles, having a distribution that correlates with primarily active H\(^+\) transport, are components of a membrane-bound H\(^+\)-ATPase (19, 20).

2. pH gradients above single mitochondria-rich cells

When toad skin epithelium is exposed to amiloride on the external surface, the short-circuit current is not brought to zero but is reversed (176a). Isolated epithelia prepared with collagenase generated short-circuit...
currents equal to $-881 \pm 68$ nA/cm$^2$, i.e., within the same order of magnitude as the active Cl$^-$ current of whole skins ($-1.630 \pm 256$ nA/cm$^2$) calculated from $^{36}$Cl$^-$ fluxes. The negative short-circuit current was reversibly reduced by brief exposure of the preparation to CO$_2$-free Ringer, and it could be abolished by adding the H$^+$-ATPase inhibitor oligomycin (10 $\mu$M) to the mucosal bath. Such a low concentration of oligomycin did not affect the active Na$^+$ current of preparations not pretreated with amiloride, indicating that oligomycin is not inhibiting mitochondrial ATP production but is interacting directly with an apical proton pump. These findings are in agreement with conclusions derived in section VIIA) in studies based on isotope tracer fluxes, i.e., the Cl$^-$ flux across the apical membrane is coupled to exit of HCO$_3^-$, and they confirm the notion that HCO$_3^-$ is derived from a CO$_2$-consuming carbonic anhydrase-catalyzed reaction that also generates protons for an apical H$^+$-ATPase. Interestingly, preparations exposed to a Cl$^--$free solution (Cl$^-$ replaced with gluconate) also generated CO$_2$-activated negative short-circuit currents. This could be explained by assuming that HCO$_3^-$ under these conditions leaves the cell via the basolateral membrane, e.g., by diffusion in Cl$^-$ channels.

To test this hypothesis (176a), a double-barreled proton-sensitive microelectrode was positioned above a MR cell of a preparation mounted in a miniature Ussing chamber and placed on the stage of an upright microscope equipped with Nomarski optics and viewed at $\times$400 magnification. With gluconate as the major anion in the external bath a pH gradient was established above individual MR cells with an average pH of 7.16 $\pm$ 0.02 ($n = 30$) at a distance 10 $\mu$m above the apical membrane and with pH of 7.40 in bulk solution. At a distance of 10 $\mu$m above principal cells of areas free of MR cells, pH was 7.37 $\pm$ 0.02. With mucosal Cl$^-$ the pH gradient collapsed (pH = 7.43 $\pm$ 0.01, 10 $\mu$m above MR cells). The measured pH gradient was used for estimating single-cell proton fluxes, which turned out to be $\sim 1.7 \times 10^{-17}$ mol $\cdot s^{-1} \cdot$MR cell$^{-1}$. Thus with a density of MR cells of $10^6/cm^2$, the macroscopic short-circuit current is expected to be $-160$ nA/cm$^2$, which is comparable, within an order of magnitude, with measured currents.

These results provide compelling evidence of a rheogenic proton pump in the apical membrane of MR cells. By showing that the presence of external Cl$^-$ prevents local mucosal acidification, the results also strongly suggest that this membrane contains an apical Cl$^-$-HCO$_3^-$ exchange system that is carrying an inward Cl$^-$ flux coupled to an outward HCO$_3^-$ flux.

With a proton pump in parallel with a Cl$^-$-HCO$_3^-$ exchange system in membranes opposite to one another (Fig. 2B, bottom), we have tentatively assumed that the basolateral membrane contains chloride channels through which Cl$^-$ diffuses into the serosal bath.

Because salt-depleted frogs, having a body fluid Cl$^-$ concentration of $\sim 100$ mM, are able to take up Cl$^-$ from 0.01 mM external Cl$^-$ solutions (161), the chemical potential difference to be overcome is 22.2 kJ/mol. In these experiments by Krogh (161) the transepithelial electrical potential difference was not measured, accordingly the total thermodynamic work is unknown. However, with in vivo transepithelial potentials between $-60$ and $+60$ mV measured in a later study (137a) and with a free energy of ATP hydrolysis of 60 kJ/mol, a H$^+$:ATP stoichiometry of 2:1 would allow for cutaneous uptake of Cl$^-$ under conditions prevailing in Krogh's classic studies. Direct studies of proton secretion against imposed electrical and H$^+$ concentration gradients indicated a ratio of 2-3 H$^+/ATP hydrolyzed (61).

### 3. γ-Type of mitochondria-rich cells

With a proton pump as well as anion transport systems in the apical membrane, the MR cells of toad skin investigated by us are clearly distinguished from the so-called α-type and β-type MR cells of distal renal epithelia having the proton pump and the anion exchange in membranes opposite to one another (Fig. 21; discussed in detail in sect. VIII). The first mentioned type of MR cell, specialized for rheogenic Cl$^-$ transport, is denoted the γ-type of MR cells. Another distinguished feature of γ-type cells is that they contain a significant apical Na$^+$ conductance (sect. IVCl).

The interdependence of passive Cl$^-$ flow and anion exchange has not been clarified. However, our studies indicated a method for separating experimentally the two functional states of the apical membrane. Short-circuiting or voltage clamping to $V = 0$ mV virtually eliminates the transepithelial Cl$^-$ conductance of $B.\ bufo$ skin (see Figs. 3 and 4A), whereby electrically silent exchange becomes the dominating mechanism for anion transport (see Fig. 5). Furthermore, with amiloride in the apical bath, the small active Cl$^-$ flux can be measured as the residual short-circuit current. Another way of eliminating the apical Cl$^-$ conductance is to reduce mucosal Cl$^-$ concentration to a few millimoles per liter (109, 142, 147, 157, 163). This of course is the condition approaching the animal in the pond where cutaneous net uptake of Cl$^-$ is not due to electrodiffusion but is depending on coupling of Cl$^-$ flow to cellular energy metabolism.
Higher densities of cells were counted in animals kept in distilled water and in KCl solutions, and in the latter experimental group the pit area was also increased. Under open-circuit conditions, the influx of Na⁺ increased along with the induced increase of MR cell pit area per square centimeter of whole epithelium, and because the proton excretion rate was increased as well, it was concluded that the proton pump is located in the apical membrane of MR cells. It was speculated that activation of this mechanism plays a role in stimulating uptake of Na⁺ from low concentrations of its salt (62a).

C. Mitochondria-Rich Cell Types Specialized for Other Functions

Vanatta and Frazier (322) showed that frogs imposed with an NH₄⁺-induced metabolic acidosis increase the rate of cutaneous acid secretion and that HCO₃⁻-induced alkalosis results in cutaneous alkalinization of the outside bathing solution. The number of MR cells of the skin increased in response to induced metabolic acidosis (247). These observations indicate that the skin, like distal renal epithelia (sect. VIII), is specialized for exercising body loads of nonvolatile acid and base and that this task is performed by the MR cells.

Ehrenfeld and Harvey (62a) induced metabolic acidosis in frogs by keeping the animals in 50 mM KCl solutions for 3–4 days. Under open-circuit conditions the isolated skin of such animals exhibited a significantly enhanced uptake of Na⁺ accompanied by increased proton secretion when compared with preparations of tap water-adapted frogs. Deoxycorticosterone acetate (DOCA) treatment of the animals as well as acidification of the serosal bath with 5% CO₂ had similar stimulatory effects on cutaneous transport of Na⁺ and H⁺.

In this study, the morphology of MR cells was also examined by counting MR cell density and measuring cross-sectional area of MR cells just below the corneum (MR cell pit area). Highest densities of cells were counted in animals kept in distilled water and in KCl solutions, and in the latter experimental group the pit area was also increased. Under open-circuit conditions, the influx of Na⁺ increased along with the induced increase of MR cell pit area per square centimeter of whole epithelium, and because the proton excretion rate was increased as well, it was concluded that the proton pump is located in the apical membrane of MR cells. It was speculated that activation of this mechanism plays a role in stimulating uptake of Na⁺ from low concentrations of its salt (62a).

The study by Ehrenfeld and Harvey (62a) confirms that frogs intentionally imposed with a metabolic acidosis develop MR cells of increased area for cutaneous elimination of protons. This type of MR cell might be identical with the so-called α-type of MR cell of distal renal epithelia, which exhibits similar morphological changes in response to metabolic acidosis of the animal (Fig. 21).

There are hints in the literature that frogs, in contrast to toads, not being intentionally acid loaded, also develop the α-type of MR cells. Thus Emilio and Menano (65) found that isolated toad skin (B. bufo) did not secrete titratable acid when exposed to NaCl Ringer on the outside. Replacing external Cl⁻ with SO₄²⁻ revealed a proton efflux of ~10 pmol·cm⁻²·s⁻¹ (65). Similar experiments with frog skin (R. ridibunda) revealed acid transport toward the apical solution of ~14 pmol·cm⁻²·s⁻¹, independent of the major anion of the external solution being Cl⁻ or SO₄²⁻ (65). Seen in retrospect, these results by Emilio and Menano are important, indicating that frogs kept under conventional laboratory conditions (running tap water at 8–10°C) develop a majority of epidermal MR cells apparently configured as α-cells of distal renal epithelia.

VIII. DISTAL RENAL EPITHELIA

High-resistance epithelia of osmoregulatory organs exhibit similar structural heterogeneity. A minority cell type resembling the MR cell in amphibian skin is found also in vertebrate urinary bladder (29) and collecting duct of the vertebrate nephron where they are denoted intercalated cells (5, 118, 254). In these epithelia the minority cells have been associated with acid-base transport and electroneutral chloride absorption. Active transport of Na⁺ has been localized to principal cells.

A. Vertebrate Urinary Bladder

Amphibians use their urinary bladder for water storage, and the epithelial cells are provided with transport systems for rendering urine sodium and chloride concentrations low (162, 177, 179, 284). Likewise, mammalian urinary bladder has the capacity to modify urine...
sodium, potassium, and chloride concentrations and to maintain low urine concentrations of sodium and chloride (188). Studies discussed here of the cellular mechanisms of these transports are based mainly on amphibian and rabbit urinary bladder. Our present understanding at the cellular level of acid-base transport by distal renal epithelia is based predominantly on studies with reptilian (turtle) urinary bladder (270, 290, 291). However, turtle bladder also reabsorbs Na⁺ (145), and the urinary bladder of the toad has the capacity of lowering luminal pH (88, 205, 206). We have no reasons to believe that species belonging to the three different classes of vertebrates differ with respect to the cellular mechanisms of transport of small ions in distal urinary epithelia. The bladder is multilayered with two or three epithelial cell layers (29, 255). Four cell types have been described (79) with the following relative numbers (% of total cell number counted on apical surface in toad bladder): large polygonal granular cells with few and short microvilli, 80%; MR cells with densely packed longer microvilli on the apical membrane, 9%; goblet cells with long filamentous microvilli, 11%; and basal cells (29, 87). Granular and basal cells are often (and in the following) denoted principal cells. From a functional point of view, three highly specialized cell types have been identified for the major transport processes (Fig. 21): active sodium uptake in principal cells, proton secretion by α-type MR cells, and bicarbonate secretion by β-type MR cells.

1. Principal cells

The lumen-negative transepithelial potential is generated by an active inward flux of Na⁺, according to the model proposed for tight epithelia (147). Sodium ions are entering the apical membrane through amiloride-sensitive channels and are actively transported from cell to interstitial fluid by an ouabain-sensitive Na⁺-K⁺ pump with a Na⁺:K⁺ stoichiometry of 3:2 (57, 58, 93, 178, 179, 189, 192, 193).

1) APICAL MEMBRANE CONDUCTANCES. A careful study with microelectrodes by Frömter and co-workers (121) of Necturus bladder revealed a serosal membrane potential of about -90 mV, irrespective of the rate of active transepithelial Na⁺ transport. Bladders with low transepithelial potentials generated a troughlike potential profile, whereas a staircase-like potential profile was found in bladders with transepithelial potentials more negative than -90 mV (121). In low- as well as in high-resistance preparations, luminal amiloride or replacement of Na⁺ by choline⁺ increased the resistance of the apical membrane to ~220 kΩ·cm². Apical membrane resistance was low in preparations generating a large short-circuit current, whereas the estimated shunt resistance was not correlated with the short-circuit current. These results imply that the spontaneous variation of the transepithelial resistance is caused by a variation of apical Na⁺ permeability with little variation of the shunt conductance among preparations (98). Such a relationship was found also in rabbit bladder, but membrane potentials of this epithelium were reported to be significantly smaller than those of amphibian bladder (189). Amiloride-sensitive sodium channels account for most of the conductance of the apical membrane (93, 189). There is evidence for two sets of such channels, one with a $P_{Na}/P_K$ selectivity of no more than 2-3 and the other being the classic channel with a $P_{Na}/P_K$ selectivity of at least 30:1 (191, 192). The Na⁺ current carried by the highly selective Na⁺ channel exhibited a dependence on external sodium and membrane voltage (194, 250), which is in agreement with the first descriptions of frog skin (95, 200, 342; see also Refs. 41 and 273). Aldosterone regulates apical sodium conductance of principal cells (37, 121, 189, 282) by increasing the number of electrically active, highly Na⁺-selective channels, possibly by recruitment from a pool of electrically dormant channels (250; for review see Ref. 249). The cellular mechanism of K⁺ secretion depends on amiloride-insensitive apical K⁺ channels in principal cells. One type of K⁺ channel is activated by membrane depolarization with time constants on the order of a few milliseconds. Aldosterone, antidiuretic hormone, and CAMP stimulate this K⁺ conductance, which is tetracethyleneammonium insensitive but Ba²⁺ and quinidine blockable (248). Apical membrane potassium currents are also carried by nonselective, oxytocin stimulated cation channels. At physiological luminal Ca²⁺ concentrations they are silent, but they become activated if luminal Ca²⁺ concentration is reduced to low values with a 50% conductance inhibition at 2.5 μmol/l luminal Ca²⁺ (2, 324, 325). The physiological significance of the nonselective cation channels is not known.

Macknight (209) has shown that Cl⁻ exchanges little, if at all, across the apical membrane of principal cells. Apical anion channels of this cell type have not been reported, and the above-mentioned analyses of the macroscopic conductance indicate that the apical membrane does not contain a significant chloride conductance.

II) BASOLATERAL MEMBRANE CONDUCTANCES. The ion selectivity of the basolateral membrane of amphibian bladder is different from that of the mammalian bladder. With a serosal membrane potential of amphibian bladder of ~90 mV (121) and chloride and potassium equilibrium potentials of about -30 and -95 mV (258), respectively, chloride conductance (and sodium conductance) is expected to be small compared with potassium conductance. In agreement with this notion, transferance numbers (T) were estimated to be $T_K = 0.71$ and $T_{Cl} = 0.04$ (42). From the membrane potential response to replacement of serosal Na⁺ with K⁺ at constant KCl product, the $P_{Na}/P_K$ selectivity of serosal membrane of rabbit urinary bladder was estimated to be ~0.044 (193). On the basis of measured intracellular activities of the three diffusible ions, a $P_{Na}/P_K = 1.17$ was then calculated to account for the relatively small serosal membrane potential of about ~90 mV (constant field
assumptions, pump current ignored (193)). The macroscopic $K^+$ conductance of urinary bladder has been associated with a $Ba^{2+}$-blockable $K^+$ channel with a $P_{Na}/P_K$ selectivity $\approx 0.1$, which displayed an increasingly open-state probability when the membrane was depolarized (190). The channels constituting the macroscopic anion conductance have also been studied using patch-clamp technique (108). Two channel types were identified both being equally permeable to $Cl^-$, $Br^-$, $I^-$, $NO_3^-$, and $SCN^-$ and sensitive to DIDS. One type of channel spent most of the time in open state at membrane potentials more positive than $-80$ mV and was reversibly closed by shifting $V$ to values more negative than $-80$ mV. This channel displayed inward rectification with a slope conductance of 64 pS between $-40$ and $-60$ mV (150 mM KCl in bath and pipette). The other channel of 362-pS unit conductance was active at membrane potentials between $\pm 20$ mV. The voltage dependencies of the two types of anion channels suggest that it is the 64-pS channel that is responsible for the macroscopic membrane $Cl^-$ conductance. With the measured channel selectivity of $P_{Na}/P_{Cl} = 0.043$ and a macroscopic membrane permeability ratio of $P_{Na}/P_{Cl} = 0.044$ (193), the 64-pS channel also accounts for the $Na^+$ leak current across the basolateral membrane of the rabbit urinary bladder (108). The polarity of its voltage dependence and its poor anion selectivity might indicate that the 64-pS channel is related to the apical $Cl^-$ channel of the MR cells and basolateral $Cl^-$ channel of the principal cells of amphibian skin.

III) INTRACELLULAR ION CONCENTRATIONS. Electron-microprobe analysis (52, 258) and studies with ion-selective microelectrodes (57, 192, 193) agree in showing that cellular $Na^+$ concentration is similarly low and intracellular $K^+$ concentration is similarly high to what is known from other epithelial tissues. These concentrations are maintained by an ouabain-sensitive mechanism in the basolateral membrane. In toad urinary bladder a ouabain-induced increase of intracellular $Na^+$ concentration could be prevented by replacing luminal $Na^+$ by choline$, implying that the cellular sodium pool of toad bladder principal cells derives $Na^+$ from the mucosal solution with insignificant recycling of this ion across the basolateral membrane (258). A similarly clear result was not obtained with rabbit urinary bladder, where application of ouabain to the serosal side resulted in cellular $Na^+$ accumulation irrespective of the presence of $Na^+$ in the mucosal solution (52). Accordingly, a $Na^+$ entrance mechanism seems to be present in the basolateral membrane. In line with this suggestion, a ouabain-induced cellular $Na^+$ accumulation could be prevented if the serosal bath was made $Na^+$ free (52). The transport system has not been identified. The intracellular $Cl^-$ concentration in toad urinary bladder was 25 mmol/kg wet mass, increasing to 56 mmol/kg wet mass in the presence of ouabain, indicating ouabain-induced cell swelling. In urinary bladder of rabbit, $Cl^-$ was close to equilibrium across the basolateral membrane, which is furnished with a relatively large $Cl^-$ permeabil-

IV) EVIDENCE FOR SYNCYTIAL-LIKE ORGANIZATION. Under control conditions, the $Na^+$ concentrations were equally low and the $K^+$ concentrations equally high in superficial and basal cells, as well as in MR and goblet cells of toad urinary bladder. Perturbation of the cation concentrations by ouabain in the presence and absence of mucosal sodium, respectively, showed that the sodium pool of basal cells depends on the exchange of sodium across the apical membrane of the outermost principal cells. Under these conditions then, sodium diffuses from one cell layer to another via intercellular junctions (258). One hour of ouabain exposure was not sufficient to allow for complete equilibration of sodium between cells of the uppermost and basal layers. Increasing the rate of active $Na^+$ transport by clamping the transepithelial potential at mucosa-positive potentials resulted in the swelling of the outermost principal cells of toad urinary bladder (8 of 12 preparations responded in this way). This response was prevented by prior exposure to luminal amiloride (17) and was enhanced by vasopressin (46). Swelling of basal cells was not seen, indicating that gap junctions restrict diffusion of $Na^+$ between uppermost and basal cells under conditions of increased $Na^+$ influx across the apical membrane. A study with microelectrodes of the intracellular voltage response to intracellular current injection con-
cluded that in rabbit urinary bladder there is a horizontal cell-to-cell coupling within the apical cell layer with no detectable electrical coupling between the cell layers (189).

The conclusion is that under certain conditions the basal cells, not being in contact with the luminal fluid, exchange their Na⁺ pool via amiloride-sensitive channels of the apical cell layer. There is doubt, however, as to what extent the basal cells contribute to the transport of Na⁺ across the bladder epithelium. The ouabain-induced changes of intracellular cation concentrations (258) and the voltage clamp-induced volume perturbations (46) provide evidence of a nature similar to that for amphibian skin in that the MR cells are not coupled to the cells of the apical layer of principal cells. In experiments with the bladder, volume changes were not estimated in vitro but by examination of fixed and sliced preparations.

2. Mitochondria-rich cells

The ionic mechanism of urinary acidification by tight epithelia has been studied extensively by Steinmetz and co-workers, using as an experimental model the urinary bladder of the freshwater turtle, *Pseudomys scripta.* Next is a discussion of the evidence that luminal acidification and alkalization are due to highly specialized cells interspersed in the epithelium: a proton-secreting α-type MR cell and a HCO₃⁻-secreting β-type MR cell. The functional organization of an acid-secreting cell suggested in early 1970s (278, 279, 290) contained the major components of the models depicted in Figure 21.

1) Rheogenic Proton Transport. When bathed in Ringer solutions on each side, isolated turtle bladders acidify the luminal solution (270) while the serosal solution becomes alkalized (289). Under open-circuit conditions of 20–60 mV luminal-negative transepithelial potentials, large pH gradients are built up of sometimes >8 pH units, showing that transepithelial proton distribution is maintained far from thermodynamic equilibrium. Similar pH gradients were recorded under short-circuit conditions. In the absence of luminal Na⁺, the (reversed) short-circuit current turned out to be equivalent to the rate of H⁺ secretion measured by the pH-stat technique, providing evidence that acid secretion is the result of rheogenic transport of protons rather than electroneutral Na⁺-H⁺ exchange. This hypothesis also accounted for the proton flux being stimulated by Na⁺ absorption under open-circuit conditions: the generated luminal-negative transepithelial potential difference increases the driving force of the outward going proton current (290, 294). The proton-transporting mechanism is coupled to cellular energy metabolism (6) and exhibits H⁺-ATPase-like activity (20, 47, 293). Andersen et al. (3) constructed a kinetic model of the proton pump accounting for the nonlinear (saturating) dependence of proton pump current on imposed transmembrane electrochemical potential difference for H⁺, with the latter quantity being manipulated either by voltage clamping or by pH perturbations.

Several methods have been used for the localization of the active proton-transporting system. Gluck et al. (108) showed that MR cells of the turtle bladder contain vesicles that take up and accumulate the weak base acridine orange, which is a fluorescent pH probe. At increasing concentrations the dye forms dimers and multiorder multimers associated with a change of its color from green at low concentrations to red-orange at high concentrations. Only MR cells contained orange vesicles, and the color disappeared after the addition of a proton ionophore, indicating dye accumulation due to low pH rather than to binding. Perfusion of CO₂-enriched Ringer solution resulted in fusion of the vesicles with the apical membrane and the discharge of dye into the luminal medium. With the impermeant large-molecular-weight dextran labeled with fluorescein isothiocyanate, intravesicular pH was estimated to be as low as 5 (105). This phase marker was taken up from the luminal bath by vesicles in MR cells when the bladder was perfused with CO₂-free Ringer solution, and it was released again to the luminal solution in response to CO₂-stimulated proton secretion. The time course of H⁺ current activation was similar to that of appearance of dextran in the luminal perfusate. Dextran release as well as H⁺ current stimulation were prevented by treatment with 0.1 M colchicine, and intravesicular pH increased in response to inhibition of cellular metabolism by iodoacetate or cyanide. Thus these studies demonstrated proton pump containing vesicle membranes that maintain low intravesicular pH. Activation of acid secretion is associated with exocytosis whereby the proton pumps are being incorporated into the luminal membrane. There is evidence that CO₂-induced exocytosis is preceded by intracellular acidification, which in turn increases cytosolic Ca²⁺ activity, and that this later event is the signal for membrane fusion (28). Endocytosis probably removes H⁺ pumps from the luminal membrane under conditions of reduced acid secretion (253). In another type of study (130, 298, 299), MR cells of fixed preparations were analyzed by carbonic anhydrase histochemistry or electron microscopy, and membrane turnover was estimated morphometrically by counting cytoplasmic vesicles and by measuring luminal membrane area. Two types of carbonic anhydrase-containing (MR) cells were identified. The α-type cell is defined as having luminal microplicae and numerous vesicles beneath the luminal pole. The cytoplasmic surface of its luminal membrane is covered with globular particles ("studded coating"), which is also found on the cytosolic side of a population of luminal vesicles ("studded vesicles"). After exposure to 5% CO₂, the number of vesicles decreased, and the luminal membrane area increased. Expansion of the apical membrane by CO₂ exposure was followed by an eight to nine times increase of outward proton flux. These cells were further characterized by having a large number of rod-shaped intramembrane particles in the apical membrane (299, 300). There was,
however, a cell type also exhibiting strong carbonic anhydrase activity, which showed little or no change in luminal membrane area in response to CO₂ exposure. These cells, denoted β-type MR cells, have simple individual microvilli on the luminal membrane, they have basal infoldings, and the studs are on the contraluminal membrane. These cells contain rod-shaped intramembrane particles, not in the luminal membrane but in the basolateral membrane. Thus the morphometric analyses of Steinmetz, Stetson, and co-workers (299, 300) also indicated CO₂-stimulated proton secretion associated with cytoplasmic vesicles fusing with the apical membrane and resulting in an increased number of pumps available for apical active proton transport. Furthermore, they showed that this process is confined to a subpopulation of MR cells, which they denoted α-cells. The other type of MR cells, denoted β-cells, were associated with bicarbonate secretion (297).

II) CHLORIDE-BICARBONATE EXCHANGE. The presence of a Cl⁻-HCO₃⁻ countertransport system in the basolateral membrane has been investigated using two types of experimental approaches. First, serosal addition of SITS inhibited proton secretion and increased cytosolic pH (32); removal of Cl⁻ from the serosal solutions also abolished proton secretion (83). Bilateral anion replacement studies showed that proton secretion, measured by short-circuiting technique after elimination of active sodium absorption with ouabain, is supported by a several anion species according to the following ranking order [(129), relative short-circuit current in parenthesis]: Cl⁻ (1) > Br⁻ (0.82) > isothiocyanate⁻ (0.52) > SO₄²⁻ (0.34), gluconate⁻ (0.34) > NO₃⁻ (0.18). These observations are compatible with coupling of apical H⁺ secretion into the luminal bath with basolateral HCO₃⁻ exit in exchange for an anion, preferentially a halide ion, of the serosal bath. Second, a membrane protein of epithelial cells from turtle urinary bladder was selectively precipitated with antibodies to both human and turtle erythrocyte anion exchanger (53). This band 3 analogue was found in ~80% of the MR cells where it was confined to the basolateral membrane. It seems correct, therefore, to assume that the SITS sensitivity and serosal anion dependence of the H⁺ current reflect interactions with a basolateral HCO₃⁻-anion exchange system of α-cells rather than a SITS-inhibitable anion-controlled HCO₃⁻ channel. [Under conditions of eliminated H⁺ secretion, the remaining basolateral Cl⁻ and HCO₃⁻ fluxes are SITS insensitive but mutually dependent (128). The anion transport pathways carrying these fluxes have not been further characterized.]

As mentioned, it was suggested that bicarbonate secretion by turtle bladder is being performed by both cells having the proton pump in the basolateral membrane and the anion exchanger in the apical membrane (297). In line with this hypothesis HCO₃⁻ secretion depends on metabolism (244) and luminal Cl⁻ (180).

III) ACTIVE TRANSPORT OF CHLORIDE. With a Cl⁻-HCO₃⁻ exchange mechanism in the luminal membrane and a chloride pump in the basolateral membrane, the β-cell is configured also for carrying an active Cl⁻ influx, energized by the basolateral H⁺-ATPase. It is worth noting that with this particular configuration of the transport systems, the active uptake of Cl⁻ is predicted to be electrically silent. The β-cell, however, cannot account for all of the active influx of Cl⁻ that contains a significant rheogenic component, as was found by Brodsky and colleagues (18, 55a, 104).

IV) IS THERE AN APICAL CHLORIDE CONDUCTANCE? The models of the acid- and base-secreting MR cells (Fig. 21) both contain a basolateral Cl⁻ conductance. The presence of an apical Cl⁻ conductance, as well, would allow for conductive flow of anions through MR cells. In this connection it is interesting that cAMP (and isobutylmethylxanthine) was shown to activate an anion system conducting Cl⁻ and HCO₃⁻ that could be blocked by acetazolamide (297). The isobutylmethylxanthine-activated anion conductance was also inhibited by luminal application of the chloride channel blocker 9-anthroic acid, and it was suggested to reside in the apical membrane of β-cells (297). It is possible then that β-cells, under certain conditions, constitute a pathway conducting transepithelial flows of Cl⁻ moving along its electrochemical potential gradient.

In a study of tightly sealed preparations of amphibian urinary bladder exposed on each side to identical NaCl Ringer solutions, the lumen-negative transepithelial potential varied from -17 to -175 mV with a mean of ~92 mV (Necturus maculosus), ~68 mV (Amphiuma tridactylum), and ~85 mV (B. marinus). Preparations of high potentials were characterized by low transepithelial resistance (Rₜ) and vice versa, however, governed by the apical membrane's sodium conductance. Resistances recorded under control conditions could be as high as 75 kΩ cm², and Rₜ values on the order of 100 kΩ cm² were recorded in bladders exposed to amiloride on the luminal side (93, 120). Rabbit urinary bladder exhibited similar dependence of transepithelial resistances on apical membrane sodium conductance with Rₜ up to 78 kΩ cm² in amiloride-exposed preparations [~1 μF/cm² apical surface area (189)]. The junctional ion permeability of rabbit urinary bladder was further investigated by measuring the unidirectional fluxes of Na⁺ and Cl⁻ (188). The efflux of Na⁺ was not reduced in the presence of luminal amiloride, and it showed no correlation with the short-circuit current, indicating that it was confined to a paracellular pathway. The calculated Na⁺ permeability of this pathway was PNa = 1.1 × 10⁸ cm/s with an associated partial ion conductance GNa = 5.0 μS/cm². Flux-ratio analysis of unidirectional Cl⁻ fluxes under open-circuit conditions indicated that the Cl⁻ fluxes obey the Ussing equation. It was also found that the Cl⁻ permeability calculated from the serosa-lumen unidirectional flux of short-circuited preparations was in rea-
sonably good agreement with the permeability calculated on the basis of the net flux of Cl\(^-\) under open-circuit conditions. These observations are compatible with the hypothesis that the fluxes of Cl\(^-\), under the conditions tested, do not contain exchange and active components. The permeability of this pathway, assumed to be paracellular, was \(D_0 = 1.5 \times 10^{-8} \text{ cm/s, with a partial Cl}\(^-\) conductance \(G_{\text{Cl}} = 7.6 \mu S/\text{cm}^2\). Thus with NaCl Ringer bathing both sides of rabbit urinary bladder, the junctional membrane resistance \(R_{\text{m}} = 79,000 \Omega \cdot \text{cm}^2\). On the basis of the estimates given above for the bladder and those given for amphibian skin (see sect. v), it appears that junctional membranes of these two high-resistance epithelia have similar conductive properties.

Passive sodium and chloride fluxes of considerably larger magnitudes than those discussed have been reported. For example, preparations of toad urinary bladder bathed with NaCl Ringer on both sides exhibited permeabilities for Na\(^+\) and Cl\(^-\) of \(\sim 10^{-6} \text{ cm/s} \) (82). These permeabilities were significantly increased by making the luminal bathing solution hypertonic, presumably due to opening of a paracellular conductance of poor selectivity (82), similar to what was described for frog skin (see sect. v).

B. Collecting Duct of Vertebrate Kidney

The collecting duct (CD) extends from the connecting segment of the distal convoluted tubule to the papillary tip and is subdivided into cortical collecting duct (CCD), outer medullary collecting duct (OMCD), and inner medullary collecting duct (IMCD). The function of the collecting duct is to reabsorb Na\(^+\), Cl\(^-\), HCO\(_3\), H\(_2\)O, and urea and to secrete K\(^+\), H\(^+\), and HCO\(_3\)\(^-\) (4, 105, 119, 122, 221, 246). The collecting duct is a single-layered epithelium of principal and intercalated cells. In some segments the intercalated cells constitute a much larger fraction in the epithelium than MR cells of amphibian skin and bladder. For example, intercalated cells of rabbit CCD, with values of \(\sim 10\) (Na\(^+\)), 127 (K\(^+\)), and 25 (Cl\(^-\)) mmol/kg wet mass (269).

1. Sites of transepithelial sodium and potassium fluxes

Isolated, microperfused CCD of the rabbit kidney develops a lumen-negative transepithelial potential of \(-15\) to \(-85\) mV. Luminal amiloride results in reversal of \(V\) and inhibition of the Na\(^+\) and K\(^+\) fluxes (304). With the microelectrode technique applied to isolated perfused segments of rabbit OMCD, two cell types could be distinguished by their serosal membrane potentials (133, 149, 149a, 150, 227). One population of cells, thought to be the principal cells, generated serosa membrane potentials of about \(-70\) mV. These cells hyperpolarized when amiloride was added to the luminal perfusion solution. This cell type is also the site of tubular secretion of K\(^+\) via Ba\(^2+\)-blockable apical K\(^+\) channels (150, 151, 227, 245, 246, 271, 272, 287). Mineralocorticoid hormones (DOCA, aldosterone) regulate K\(^+\) secretion by activating the apical K\(^+\) conductance preferentially in the (CCD) with little effect on OMCD, and OMCD\(_i\) (152). The other population of cells that could be distinguished by electrophysiological criteria generated serosa membrane potentials of no more than \(-20\) to \(-40\) mV, the fractional resistance of their apical membrane was near unity, and they did not respond to luminal amiloride. These cells were thought to be intercalated cells (133, 143, 149a, 150, 227). The small intracellular potential of these cells and the lack of measurable apical membrane (Na\(^+\)) conductance suggest a low K\(^+\) conductance in the serosal membrane as well. In agreement with this notion the intracellular potential of this cell type did not depolarize in response to stepping the serosal K\(^+\) concentration from 5 to 50 mM (149, 150) or the addition of Ba\(^{2+}\) to the serosal bath (227).

Steady-state intracellular concentrations of Na\(^+\), K\(^+\), and Cl\(^-\) are similar in identified principal and intercalated cells of rabbit CCD, with values of \(\sim 10\) (Na\(^+\)), 127 (K\(^+\)), and 25 (Cl\(^-\)) mmol/kg wet mass (269). Functionally the two cell types could be clearly distinguished, however, by different responses of cellular cation concentrations to ouabain. In principal cells, a 10-min exposure to ouabain induced an increase in cell sodium to >100 mmol/kg wet mass and a decrease in cell potassium to \(\sim 30\) mmol/kg wet mass. In intercalated cells the sodium concentration responded with a much smaller increase to \(\sim 34\) mmol/kg wet mass, and cell potassium showed a correspondingly smaller decrease to 105 mmol/kg wet mass. The ouabain-induced cation concentration changes of principal cells were signifi-
cantly reduced by application of amiloride to the luminal perfusion solution (10\(^{-4}\) M) and the serosal bath (10\(^{-3}\) M). When Na\(^+\) was removed from the serosal bath, in the presence of luminal amiloride, cell Na\(^+\) dropped to \(\sim 5\) mmol/kg wet mass. Taken together these results indicate that apical Na\(^+\) channels as well as basolateral Na\(^+\)-H\(^+\) antiporters mediate fluxes of Na\(^+\) into the principal cells. The above K\(^+\) concentrations of rabbit CD under control conditions agree reasonably well with the K\(^+\) activity measured with a potassium-selective electrode [94 mM (267)]. In cells of *Amphiuma* kidney CD the
intracellular sodium activity (~8 mM) and potassium activity (~56 mM) are both maintained at nonequilibrium values by the operation of a basolateral Na⁺-K⁺ pump, an amiloride-blockable sodium path in the apical membrane, and a relatively large K⁺ conductance in the basolateral membrane (196). The ouabain-induced volume response also differs among principal and intercalated cells. Using video-enhanced microscopy, Strange (305) measured the volume of identified cells of microperfused CD of DOCA-treated rabbits. Although principal cells started swelling immediately in the presence of 10⁻⁴ M serosal ouabain, the volume of intercalated cells stayed virtually constant over an observation period of 5 min. Ouabain-induced cell swelling of the principal cells could be prevented by amiloride in the luminal perfusion solution.

The general conclusion is that principal cells of the collecting duct are configured for active cation transport according to the classic model for high-resistance epithelia (147). The K⁺ secretion is also mediated by these cells having a K⁺ conductance in the apical membrane, and it is the Na⁺-K⁺-ATPase in the serosal membrane that provides energy for the active transport of the two alkali ions. It can also be concluded that intercalated cells exhibit a slow turnover of intracellular alkali ions. It is not likely that they play a major role in translocating Na⁺ and K⁺ across the epithelium.

The agreement of 1) different intracellular potentials of the two cell types under control conditions, 2) the different voltage responses of the two cell types to serosal K⁺ steps or serosal Ba²⁺ exposure, and 3) their different electrolyte and volume responses to serosal application of ouabain indicates that principal and intercalated cells do not communicate with one another via low-resistance gap junctions.

2. Sites of acid-base transports

Stoner et al. (304) showed that amiloride-induced reversal of the transepithelial potential of CCD could be prevented either by adding acetazolamide or by eliminating CO₂ from the bathing solutions. These observations led to the hypothesis that the luminal-positive potential was caused by an electrogenic proton-secreting mechanism and that protons are derived from a carboxic anhydrase-catalyzed hydration of CO₂ (304). The presence of high carboxic anhydrase activity in intercalated cells (203, 263) logically associated this cell type with acid transport. During recent years this hypothesis has gained strong support.

The α- and β-type intercalated cells have been described in rats (22, 329) and rabbits (275, 276). The α- and β-cells have several features in common that distinguish them from principal cells: 1) they contain many mitochondria, 2) they are rich in carboxic anhydrase (23, 204, 262), and 3) they contain large numbers of the rod-shaped intramembranous particles (262). The two forms of intercalated cells are morphologically distinguished from one another, as α-cells are light with many apical micropileae and with many cytoplasmic vesicular bodies and β-cells have a darker cytoplasm and extensive basolateral infoldings (215). Ultrastructural analysis of the distribution of rod-shaped particles and carboxic anhydrase activity, however, indicates that intercalated cells of rabbit kidney exhibit a wider heterogeneity than MR cells of the bladder (262). The functional significance of this heterogeneity is not known.

Different segments of CD exhibit markedly different capacities for acid-base transport. Acid secretion is localized primarily to the OMCD (334), which contains almost exclusively α-cells (149). In rats with acute metabolic acidosis the surface density of the apical membrane of α-cells increased in OMCD (213, 214) and CCD (329) without detectable ultrastructural changes in the β-cells of CCD (329). Apically, the α-cells contain coated vesicles with proton pumps, which by exocytosis are incorporated into the luminal membrane in response to stimulation of acid secretion (20, 22, 275-277). Together with the demonstration of a basolateral Cl⁻ conductance (149, 149a, 227) and a band 3-like protein in the basolateral membrane (125, 334), these studies all support the model for the α-cell of the urinary bladder (Fig. 21). The β-cells of OMCD, which has a capacity for luminal alkalization (134, 202, 220, 221) by way of active Cl⁻-dependent HCO₃⁻ secretion (288), have a reversed arrangement of the H⁺-ATPase and Cl⁻-HCO₃⁻ exchange system (22, 276, 277; Fig. 21). Schwartz et al. (276) were able to reverse the functional polarity of the HCO₃⁻-secreting intercalated cells of CCD by acid-loading the animal (rabbit). This treatment resulted in a decreased number of II⁺-secreting cells (cells with an apical proton pump), with a concomitant decrease in number of HCO₃⁻-secreting cells (cells with a basolateral Cl⁻-HCO₃⁻ exchanger) without increasing the total number of intercalated cells. This study was the first indicating that unidirectional acid-base transport by intercalated cells is not due to an intrinsic polarity of a given cell type but is induced by environmental stimuli.

3. Sites of transepithelial chloride fluxes

The first study of Cl⁻ transport in isolated microperfused segments of rabbit CD (304) indicated three mechanisms of transepithelial Cl⁻ fluxes: Cl⁻ self exchange, active transport, and passive (conductive) transport.

1) PRINCIPAL CELLS. The intracellular Cl⁻ activity in CD of rats measured with ion-selective electrodes was found to be only ~10 mM (272). A similar low Cl⁻ concentration in the principal cells of rat CD was measured with electron-microprobe technique (7), which is significantly lower than the Cl⁻ concentration (25 mM) of principal cells of rabbits (269). These observations indicate species differences between rabbits and rats. In principal cells of rabbits (269), but not rats (272), the
intracellular Cl⁻ concentration is significantly above its thermodynamic equilibrium value. The transport system(s) responsible for this distribution is as yet unknown.

Amiloride induced cellular hyperpolarization was associated with an increase in the fractional resistance of the apical membrane to near unity (150), suggesting that the apical membrane of the principal cells does not contain Cl⁻ channels. Furthermore, a large Cl⁻ concentration step on the serosal side was not reflected in the steady-state basolateral membrane potential (150, 227). As judged from these studies the principal cells are relatively tight to Cl⁻. A preliminary patch-clamp study of cultured principal cells revealed an apical rectifying Cl⁻ channel of relatively large unit conductance, ~120 pS. The channel exhibited a low fractional open time, and it was seen in <1% of the channel recordings (30).

The confluent cultured epithelium developed luminally negative transepithelial potentials of ~21 to ~72 mV and was differentiated for active uptake of sodium and potassium secretion. The epithelium responded by a small hyperpolarization to apical application of the chloride channel blocker 5-nitro-2-(3-phenylpropylamino)benzoate (NPPB), indicating the presence of a (very) small apical Cl⁻ conductance (106). Furthermore, cell volume studies indicated the presence of an apical entry step for Cl⁻, as principal cells gained volume when exposed to serosal ouabain and apical (unilateral) Cl⁻ (305). This result was obtained with tubules from DOCA-treated animals, only perhaps suggesting that DOCA treatment induced an apical anion pathway. With a cellular concentration of 20–30 mM (269) and a serosal membrane potential close to ~70 mV (133, 149a, 150, 227), cellular Cl⁻ of principal cells is far above thermodynamic equilibrium. Thus the apical uptake of Cl⁻ is mediated by a channel. This apical Cl⁻ transport system, apparently activated by mineralocorticoid (DOCA) treatment of rabbits (305), has not yet been identified, and it is not known whether it generates significant transcellular Cl⁻ fluxes.

II) INTERCALATED CELLS. Investigations of Cl⁻ absorption and bicarbonate secretion by CD have indicated that it is an apical exchange transport system, related to that of the red blood cell membrane, which mediates electroneutral fluxes of Cl⁻ and HCO₃⁻ (107, 220, 221, 302, 306, 307). It has been assumed that it is the β-cell type of the CD that is specialized for electroneutral reabsorption of Cl⁻. The apical uptake of Cl⁻ associated with ouabain-induced cell swelling cannot be mediated by a channel. This apical Cl⁻ transport system, apparently activated by mineralocorticoid (DOCA) treatment of rabbits (305), has not yet been identified, and it is not known whether it generates significant transcellular Cl⁻ fluxes.

This review discusses the functional organization of epithelia consisting of more than one type of cell specialized for ion transport: amphibian skin, vertebrate urinary bladder, and collecting duct of vertebrate kidney. All of these epithelia are specialized for vectorial transport of NaCl from an external compartment to the interstitial fluid compartment. Large Na⁺ and Cl⁻ electrochemical potential gradients are either being maintained or built up. Due to relatively low passive ion conductances, the rectified flux of Na⁺ is creating a large serosa-positive transepithelial potential difference.

A. Principal Cell Compartment: Common Functional Organization

Principal cells of all three epithelia constitute the major Na⁺-transporting compartment. The amphibian...
FIG. 22. Comparison of models of ion-transporting cells of high-resistance epithelia. Principal cells are specialized for active uptake of Na⁺ energized by a basolateral Na⁺-K⁺ ATPase. Passive entry of Na⁺ across apical membrane. K⁺ secretion when apical K⁺ channels are activated. Na⁺-2Cl⁻-K⁺ cotransporter and Cl⁻ channels in basolateral membrane (frog skin) maintain intracellular Cl⁻ above electrochemical equilibrium. Normally, Cl⁻ fluxes through these pathways are small, and Cl⁻ is exchanged by an electroneutral SITS/DIDS-inhibitable anion transporter. MR cells exhibit high carbonic anhydrase (CA) activity. H⁺ and HCO₃⁻ are transported out of cell by rheogenic proton pump and anion exchanger, respectively. Cl⁻ leaves cell through channels in basolateral membrane. Energized by H⁺-ATPase, MR cells serve different functions. α-Type, acid (proton) elimination; β-type, base (HCO₃⁻) secretion and nonrheogenic Cl⁻ uptake; γ-type, rheogenic uptake of Cl⁻. This type also contains Cl⁻ channels in apical membrane (not shown). At high mucosal Cl⁻ concentration, Cl⁻ channels are activated by apical membrane depolarization. Only γ-type have been shown to contain a significant apical Na⁺ conductance.

skin is the classic preparation used by Ussing and colleagues for the first analyses associating electrical properties of the epithelium with active and passive flows of ions. The two-membrane theory of the functional organization of amphibian skin (Fig. 22) obviously applies also to the distal renal epithelia. The active Na⁺ flux generated by the principal cells is governed by common transport systems: apical amiloride-sensitive sodium channels in series with basolateral Na⁺-K⁺ pumps. The K⁺ actively transported into the cells across the serosal membrane returns to the interstitial fluid by passive flow in K⁺-selective channels. The apical membrane also contains potassium channels. They can be studied after an imposed body potassium load or by hormonal treatment. It is generally agreed that the principal cell compartment is specialized for eliminating K⁺, with the secretory flux of K⁺ being energized by the basolateral membrane’s Na⁺-K⁺-ATPase. Biophysical studies of the channels conducting basolateral and apical K⁺ currents have shown that they exhibit far more diversity than the Na⁺ channels, but the associated functions are, as yet, not well understood (249, 328). Voltage-dependent chloride channels of weak anion selectivity are present in basolateral membranes of frog skin and urinary bladder. It is indicated that the large tracer flux of Cl⁻ across the basolateral membrane is not mediated but associated with anion exchange via a DIDS or SITS-inhibitable electroneutral mechanism (301). Harvey and Ehrenfeld (112, 113) have shown that at alkaline cellular pH this mechanism accepts HCO₃⁻, and under conditions of cellular acidification an Na⁺-H⁺ exchanger in the basolateral membrane becomes activated. In amphibian skin, Cl⁻ is maintained above thermodynamic equilibrium by a slowly operating Na⁺ gradient-driven cotransport system (1 Na⁺:2 Cl⁻:1 K⁺), which is also involved in regulation of cell water volume. It is indicated that Cl⁻ is above equilibrium also in principal cells of distal renal epithelia. It remains to be defined whether the volume regulatory mechanisms of amphibian skin are operating in the distal renal epithelia.
In amphibian skin and urinary bladder, Cl⁻ does not exchange between outside and principal cell compartments, providing good evidence that principal cells do not mediate transepithelial fluxes of Cl⁻. There is no general conclusion yet on the significance of principal cells of CD for the transport of Cl⁻.

B. Mitochondria-Rich Cells: Subpopulations With Specialized Functions

The MR cells all exhibit a significant carbonic anhydrase activity. Freeze-fracture studies have disclosed a common type of rod-shaped intramembrane particle on the P-face of the plasma membrane, recently associated with H⁺-ATPase activity. Mitochondria-rich cells do not seem to communicate with principal cells via low-resistance gap junctions.

In amphibian skin, the passive flow of Cl⁻ is carried by the mitochondria-rich cells. Their apical membrane contains an external chloride-dependent and voltage-gated Cl⁻ permeability and an amiloride-blockable Na⁺ conductance. Mathematical models of the skin epithelium have been constructed that are integrating the electrophysiological description of the ionic currents carried by pumps and passive conductors of the syncytial principal cell compartment with that of an arbitrary number of much smaller MR cells containing a voltage-gated apical Cl⁻ permeability. The electrophysiological behavior of the model shows that with respect to major features the agreement with the observed behavior of the real epithelium is satisfying. This description also predicts and explains in detail the effects of blocking apical sodium channels on the time-dependent inhibition of the chloride conductance without having to assume direct interaction of sodium channel blockers with chloride channels. Computer analyses of epithelial models are superior to the conventional circuit analysis, as they are coping with nonohmic behavior of membrane conductances and with time-dependent states caused by perturbation of intracellular ion concentrations and gating variables of dynamic membrane permeabilities.

The site of conductive Cl⁻ fluxes across urinary bladder and CD epithelium has not been unequivocally identified. With regard to the CD, the favored view is that the major reabsorption of Cl⁻ driven by the transepithelial potential difference takes place along a paracellular pathway of relatively high conductance (~1 mS/cm²), exhibiting a Cl⁻:Na⁺ mobility ratio similar to a free-diffusion path (335; for review see Ref. 274). However, voltage-operated (195) and protein Gi-,-regulated (280) apical Cl⁻ channels of large unit conductance have now been found in intercalated cells in primary culture. The polarity of their voltage dependence is similar to that of apical Cl⁻ channels of MR cells of amphibian skin, where they generate the major component of the transepithelial Cl⁻ current. Similar to what was found for MR cells in toad skin epithelium, CAMP appears to activate an apical chloride conductance also in MR cells of urinary bladder.

1. α-, β-, and γ-types of mitochondria-rich cells

The heterocellular epithelia considered here also have the capacity for transepithelial acid and base transport as well as for transepithelial active transport of Cl⁻. The turtle bladder has constituted a superior experimental model for studies of the cellular mechanisms of urinary acidification by distal renal epithelia. Steinmetz and colleagues have provided evidence that in urinary bladder, acid and base secretion are due to two subpopulations, the α- and β-type MR cell, respectively. Common features of models for α- and β-cells are 1) acid and base secretions are both energized by an electrogenic H⁺-ATPase; 2) protons are derived from hydration of CO₂ catalyzed by carbonic anhydrase, resulting in cellular accumulation of HCO₃⁻; 3) HCO₃⁻ is transported out of the cell in exchange with extracellular Cl⁻; 4) Cl⁻ is leaving the cell across the basolateral membrane along its electrochemical potential gradient; and 5) the proton pump and the anion exchanger are localized in membranes opposite to each other (Fig. 22). There is now compelling evidence that the active Cl⁻ current of amphibian epidermis is also energized by a proton pump, located, however, to a third type of MR cells, the γ-cells, that have the proton pump and an anion-exchange system in the same membrane, i.e., the apical membrane (Fig. 22).

Subpopulations of cells with an ultrastructure similar to α- and β-cells and with acid-base transporting capacity are also found in CD of the mammalian kidney, and their occurrence along the duct correlates with known acid-base secretory functions of each of the CD segments. Depending on animal species, intercalated cells of kidney exhibit a greater variation in morphological heterogeneity than MR cells of the bladder, as judged by distribution of intramembrane particles and occurrence of carbonic anhydrase activity. Amphibian epidermis, like distal renal epithelium, is also excreting body loads of acid and base, and this task is performed by MR cells. Thus MR cells of amphibian skin also seem to constitute subpopulations: one type being specialized for uptake of Cl⁻ and the other types being specialized for acid and base transports.

The α-, β-, and γ-type MR cells appear not to be intrinsic to the epithelia in which they were first observed. Among scopes for future studies are questions concerning the control of their relative abundances in response to conditions prevailing during the experiment or conditions imposed on the animal before investigation.

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