Osmoregulation and Excretion

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ABSTRACT

The article discusses advances in osmoregulation and excretion with emphasis on how multicellular animals in different osmotic environments regulate their *milieu intérieur*. Mechanisms of energy transformations in animal osmoregulation are dealt with in biophysical terms with respect to water and ion exchange across biological membranes and coupling of ion and water fluxes across epithelia. The discussion of functions is based on a comparative approach analyzing mechanisms that have evolved in different taxonomic groups at biochemical, cellular and tissue levels and their integration in maintaining whole body water and ion homeostasis. The focus is on recent studies of adaptations and newly discovered mechanisms of acclimatization during transitions of animals between different osmotic environments. Special attention is paid to hypotheses about the diversity of cellular organization of osmoregulatory and excretory organs such as glomerular kidneys, antennal glands, Malpighian tubules and insect gut, gills, integument and intestine, with accounts on experimental approaches and methods applied in the studies. It is demonstrated how knowledge in these areas of comparative physiology has expanded considerably during the last two decades, bridging seminal classical works with studies based on new approaches at all levels of anatomical and functional organization. A number of as yet partially unanswered questions are emphasized, some of which are about how water and solute exchange mechanisms at lower levels are integrated for regulating whole body extracellular water volume and ion homeostasis of animals in their natural habitats. © 2014 American Physiological Society. *Compr Physiol* 4:405-573, 2014.

Introduction

In his lecture series as professor of comparative physiology at the Museum of Natural History in Paris Claude Bernard (1813-1878) wrote: "I believe I was the first to insist on the concept that animals have actually two milieus; an external milieu in which the organism is located and an internal milieu in which the elements of its tissues live. The real existence of a living thing does not take place in the external milieu, which is the atmosphere for air breathing creatures and fresh or salt water for aquatic animals, but in the liquid internal milieu formed by the circulating organic fluid surrounding and bathing all the anatomical elements of the tissue" (113). Inspired by this idea of Bernard and his own studies of glucose metabolism, body temperature and acid-base balance, Walter B. Cannon (1871-1945) developed the concept of "homeostasis." Homeostasis, Cannon pointed out, would require mechanisms operating simultaneously or successively in such a way that perturbation of a physiological variable initiates processes that would counter the change (250). In experiments with freshwater animals, August Krogh (1874-1949) discovered that their osmoregulation depends on active ion transport across the skin and gills and concluded that the different ion composition of the extracellular and intracellular body fluids, and of freshwater animals and their natural surroundings, "has not to do with a true equilibrium, but with a steady state maintained against a passive diffusion and requiring expenditure of energy" (974).

The ideas of the above seminal works are embedded in the comparative physiology of osmoregulation and excretion dealing with the regulation of composition and volume of the internal milieu of multicellular organisms exposed to different and time-varying external milieus. In order to study the regulation of fluxes of solutes and water between the animal and the surroundings, the transport systems and their energy requirement have to be identified and related to the anatomical and cellular organization of ion- and water-transporting tissues and excretory organs, which have constituted major efforts in comparative studies of osmoregulation and excretion.

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The present article is organized as follows. The initial section on "Biophysical Concepts in Osmoregulation" presents commonly used concepts and theoretical tools for the study of water and ion exchange in animals. Some mathematics is necessary in order to express the issue in a precisely defined form. This is done here by explaining the results in qualitative, biologically relevant terms of importance for animal osmoregulation. The subsequent sections are organized according to taxonomic groups to emphasize that comparative physiology of osmoregulation is about principles and diversity of adaptations that have evolved among different animal species to cope with changing osmotic environments. Our discussions include physiological and biochemical mechanisms at all levels of functional organization. It will be clear that scientific progress in different taxa proceeds along somewhat different paths. We aim at a review on the experimental background of the present ideas and at providing stepping-stones for future studies of this major and expanding field of comparative physiology. Specifically, the future challenge is to integrate whole body, isolated tissue, and cell physiological research with progress in studies on the organization of the genome and expression patterns.

Biophysical Concepts in Osmoregulation

The section summarizes quantitative treatments of water and ion transport across plasma membranes and epithelia, which are pertinent to studies and discussions of animal osmoregulation.

Chemical Potential of Water, Osmotic Pressure, and the van't Hoff Equation

Assume two compartments of water, (a) and (b), separated by a water-permeable membrane. The chemical potentials of water are given the symbols, $\mu_w^{(a)}$and$\mu_w^{(b)}$. If water is not in thermodynamic equilibrium ($\mu_w^{(a)} \neq \mu_w^{(b)}$), there is a (net) flux of water, $J_w^{(net)}$, directed from the compartment of the higher chemical potential to that of the lower chemical potential. If there is no interaction in the membrane between the water molecules or between water molecules and other moving particles, the flux-ratio equation applies (1889),

$$RT \ln \frac{J_w^{(a)\rightarrow(b)}}{J_w^{(a)\leftarrow(b)}} = \mu_w^{(a)} - \mu_w^{(b)} \tag{1a}$$

$$J_w^{(net)} = J_w^{(a)\rightarrow(b)} - J_w^{(a)\leftarrow(b)} \tag{1b}$$

where R is the universal gas constant (8.31 $J \cdot K^{-1} \cdot mol^{-1}$ or 0.082 $l \cdot atm \cdot K^{-1} \cdot mol^{-1}$) and T the absolute temperature (K). The unidirectional water fluxes ($J_w^{(a)\rightarrow(b)}$, $J_w^{(a)\leftarrow(b)}$) are measured by the isotope tracer technique using ^3H_2O, D_2O or H_2O^{18}, or by a combination of the tracer technique for determining one of the unidirectional fluxes and a volumetric measurement of the net flux of water. Water transport across biological membranes takes place in the lipid bilayer between integral proteins (532), via aquaporins constituting selective water-permeable pores (7, 8, 1483, 1484), and in the narrow tight junctions of the paracellular pathway of absorbing and secreting epithelia (906, 1892, 1984). Although the transport is passive, Eq. (1a) is rarely obeyed because bulk water flow in pores violates the assumption of random thermal movement of individual water molecules, which was discovered already in the early studies of water transport in biological systems (746, 955, 1399). Since then, it has been a major challenge for physiologists to develop a consistent theoretical framework for handling of water transport across biological membranes (532, 804).

The chemical potential of water is determined by the mole fraction of water (X_w) according to (1761),

$$\mu_w = \mu_w^O(T, p) + RT \ln X_w$$

where $\mu_w^O(T, p)$ is the chemical potential of pure water ($X_w = 1$) at temperature, T, and pressure, p. Assume that compartment (a) contains pure water and is separated from compartment (b) of similar temperature, but containing the impermeable solute, s of mole fraction X_s. Thus, $X_w = 1$ of compartment (a), and $X_w + X_s = 1$ of compartment (b). Thermodynamic equilibrium for water, $\mu_w^{(a)} = \mu_w^{(b)}$, requires,

$$\mu_w^O(T, p^{(a)}) = \mu_w^O(T, p^{(b)}) + RT \ln X_w$$

Since $X_w < 1$, $RT\ln X_w$ is negative. Thus, $p^{(b)} - p^{(a)} > 1$, which is the osmotic pressure, Π, of the solution in compartment (b) given by the following thermodynamically exact expression (1761),

$$\Pi = -\frac{RT}{\langle \overline{V}_w \rangle} \ln(1 - X_s) \tag{2}$$

where $\langle \overline{V}_w \rangle$ is the mean value of the molar water volume, $\overline{V}_w$, in the pressure range $p^{(a)}$ to $p^{(b)}$. For a dilute solution, $X_s \ll 1$ and $\ln(1 - X_s) \approx -X_s$ that leads to,

$$\Pi = \frac{RT}{\langle \overline{V}_w \rangle} X_s \tag{3a}$$

Introducing the concentration of the solute rather than its mole fraction presupposes further mathematical approximations. Since Eq. (3a) assumes the solution to be dilute, $X_s = n_s/(n_w + n_s) \approx n_s/n_w$, where n_s and n_w are the number of gram molecules of s and w. Thus,

$$\Pi = \frac{RT}{n_w \langle \overline{V}_w \rangle} n_s = RT \frac{n_s}{[V_w]} \tag{3b}$$

Here, $n_w \langle V_w \rangle = [V_w]$ is the volume occupied by water of the solution with a total volume of V. Therefore, $n_s / [V_w]$ is the volume molality, which for diluted solutions is equal to the *weight molality*, m, in moles per kilogram of water and referred to as the *osmolality of the fluid*. The *molar concentration* or the *osmolarity* of the solution is defined by, $c_s = n_s/V$, and for diluted solutions, $n_w \langle \overline{V}_w \rangle \approx V$, which, inserted into Eq. (3b), gives,

$$\Pi = RTc_s \tag{3c}$$

This equation expresses the *van't Hoff's law* (1895), with c_s being the solute concentration in moles per liter of solution. Because the volume of water is temperature dependent, the osmolarity of a solution is temperature dependent.

Estimation of osmolality of animal body fluids, colligative properties

Determination of the chemical potential of water is a prerequisite for investigating the nature of the distribution and fluxes of water between compartments in an animal and between an aquatic animal and its environment. As indicated above, in *ideal* (i.e., diluted) solutions, the osmotic pressure depends on the number of dissolved particles per unit of volume of water, but is independent of the chemical composition, size, shape, and electrical charge. A corollary to this assumption is that the forces of interactions between water molecules and between water molecules and dissolved molecules do not differ. For diluted solutions, the osmotic pressure (Eq. 2) and the osmolality (Eq. 3b) are both measures of the chemical potential of water. The osmotic pressure is one of four colligative properties. With reference to pure water, the impacts of solutes on colligative properties are: (i) increase in osmotic pressure, (ii) lowering of vapor pressure, (iii) elevation of boiling point, and (iv) depression of freezing point. Eq. (2) and Eqs. (3a-c) are valid only for dilute solutions. Biological fluids are not ideal solutions. Because there is no thermodynamically exact way of dealing with this, if the molar concentration is known, one may introduce an empirical dimensionless temperature-dependent osmotic coefficient (ϕ) as correction factor to the van't Hoff equation,

$$\Pi = \phi RTc_s \tag{3d}$$

Values of ϕ for electrolyte solutions of different concentrations are listed in ref. (1567); as an example, for c_{NaCl} = 100 mM at 25 °C, $\phi = 0.932$, and because NaCl in aqueous solution is fully dissociated, $\Pi = 0.932 \cdot 0.0821 \cdot 298 \cdot 0.2 = 4.56$ atm. Thus, as a first approximation, by adding NaCl to pure water to a final concentration of 100 mmol/l, the chemical potential of water would be lowered by $\Delta\mu_w = 0.932 \cdot 8.31 \cdot 298 \cdot 0.2 = 463$ J/l. Generally, the composition of biological fluids is not known exactly. Therefore, one of the colligative properties is measured from which the osmolality is estimated. The freezing point depression and the lowering of vapor pressure can be measured in samples of the order of 10^{-7}-10^{-6} l by commercially available instruments and are therefore preferred in studies of biological fluids. Theoretical and technical problems in using of these methods to non-ideal solutions are discussed in ref. (1795).

Transport equations

The volume flow across a membrane that is permeable both to water and solute is given by (532),

$$J_V = L_p(\Delta p - \sigma RT \Delta c_s) \tag{4a}$$

where L_p is the hydraulic conductance, Δp is the hydrostatic pressure difference between the two compartments, Δc_s is the solute concentration difference between the compartments, and σ is the dimensionless reflection coefficient defined such that $\sigma = 0$ if the membrane cannot distinguish between water and solute, and $\sigma = 1$ if only water can pass. The hydraulic conductance is related to the osmotic permeability, P_f, by (532),

$$P_f = \frac{RT}{\overline{V}_w} L_p$$

where $\overline{V}_w$ is the partial molar volume of water, which is ~ 18 cm^3·mol^{-1} at 20 °C. In animal cells with soft plasma membranes, $\Delta p \cong 0$ in Eq. (4a). Thus,

$$J_V = P_f \overline{V}_w \sigma \Delta c_s \tag{4b}$$

If the water exchange is studied using isotopes, diffusion permeabilities (P_{dw}) are obtained,

$$J_w^{(a \rightarrow b)} = \overrightarrow{P_{dw}} c_{H_2O}^{(a)}$$
$$J_w^{(a \leftarrow b)} = \overleftarrow{P_{dw}} c_{H_2O}^{(b)}$$

For an oil membrane in which the concentration of water is so low that interactions between water molecules can be neglected,

$$\overrightarrow{P_{dw}} = \overleftarrow{P_{dw}} = P_{dw} \text{ and } \frac{P_f}{P_{dw}} = 1$$

If there is a net water movement across a porous membrane, the isotope flux is accelerated in the direction of net water flow and impeded in the opposite direction, resulting in, $\overrightarrow{P_{dw}} \neq \overleftarrow{P_{dw}} \neq P_f$.

This inequality was indicated in the very first study using isotopes for investigating the nature of water transport across biological membranes (746).

Partition of Ions between Intra- and Extracellular Compartments

Exchange of water, ions, and organic molecules between the organism and the environment takes place through epithelia of the integument and the gills, and through "internal" epithelia such as gastrointestinal tract, kidney tubules, urinary bladder, and exocrine glands. The physical-chemical concepts of ion transport between extracellular fluid and the surroundings are the same as those of the exchange of ions between the cell and the extracellular fluid. The common electrolytes of body fluids are always fully dissociated into ions. In highly diluted solutions, the ions move freely without interacting with each other, so that the mass concentration, c, of an ion is the same as its activity, a. Biological fluids are not highly diluted, which means that the activity of an ion on which its kinetic and thermodynamic properties depend is different from its mass concentration, $a = f{\cdot}c$, $0 < f \leq 1$. The activity coefficient, f, depends on the valence of the ion, the ionic strength of the solution, and the dielectric constant of the solvent. The textbook by Robinson and Stokes (1567), which is frequently consulted by physiologists, discusses theories of ion-ion and ion-water interactions in fluids of different concentrations and provides tables of activity coefficients and other thermodynamic parameters of relevance for biological fluids.

Fig. 1A shows a plasma membrane that separates an intracellular from an extracellular compartment that contain the membrane permeable ion, j. Temperature and hydrostatic pressure, respectively, are assumed similar in the two compartments. The ion flux J is defined as the number of moles of j, which pass the unit area of plasma membrane per unit time. If the ion transport (or the transport of any solute) is estimated from the flux of a radioactive isotope, one denotes the isotope flux from the outside into the cellular fluid as the 'influx', $J_j^{(in)}$, while the isotope flux in the opposite direction is denoted the 'efflux', $J_j^{(out)}$. Having accounted for the specific activity of the isotope in the compartment into which it was introduced, under physiologic stationary conditions the "net flux" is calculated as, $J_j^{(net)} = J_j^{(in)} - J_j^{(out)}$.

Electrochemical potentials and thermodynamic equilibrium

The electrochemical potentials of j, $\tilde{\mu}_j$, in the two fluid compartments are:

$$\begin{aligned}\tilde{\mu}_j^{(e)} &= \mu^O + RT\ln a_j^{(e)} + z_j F\psi^{(e)} \\ &= \mu^O + RT\ln(f_j^{(e)}c_j^{(e)}) + z_j F\psi^{(e)}\end{aligned} \tag{5a}$$

$$\begin{aligned}\tilde{\mu}_j^{(c)} &= \mu^O + RT\ln a_j^{(c)} + z_j F\psi^{(c)} \\ &= \mu^O + RT\ln(f_j^{(c)}c_j^{(c)}) + z_j F\psi^{(c)}\end{aligned} \tag{5b}$$

where μ° is chemical potential at standard conditions, F is the Faraday (96485 C·mol^{-1}), and z_j is the valence of the ion. If

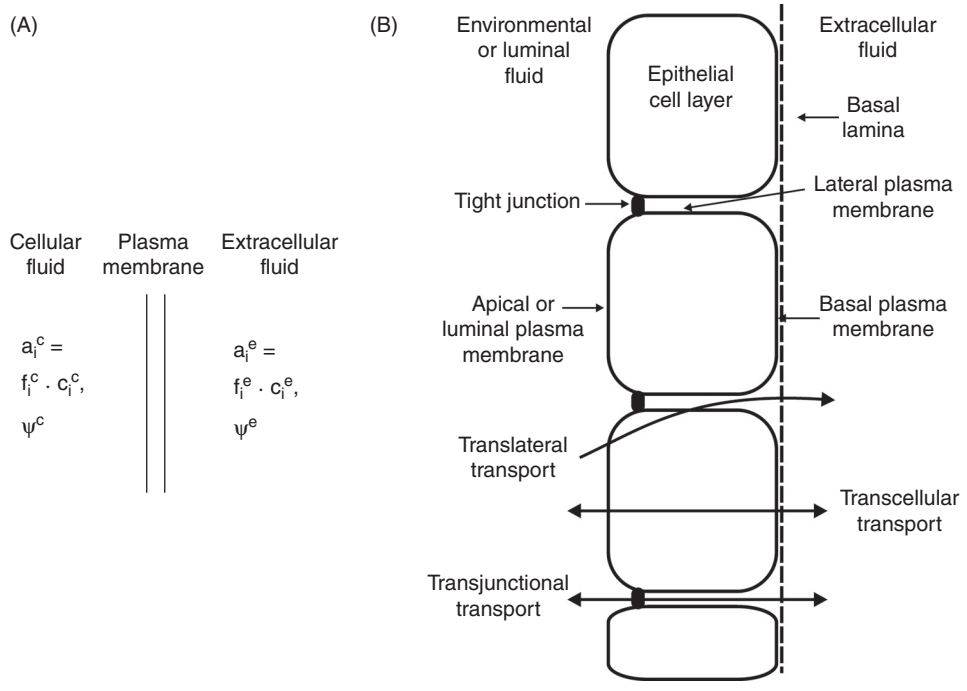

Figure 1 (A) Plasma membrane (m) separating the cell compartment (c) from the interstitial fluid compartment (e). Symbols: $\psi^{(c)}$, $\psi^{(e)}$: electrical potential of cellular (c) and extracellular fluid (e); $a_j^{(c)}$, $a_j^{(e)}$: chemical activities of j; $c_j^{(c)}$, $c_j^{(e)}$: chemical concentrations of j; $f_j^{(c)}$, f_j^e : activity coefficients of j. (B) Transepithelial pathways in epithelia.

the electrochemical potentials are identical, $\tilde{\mu}_j^{(c)} = \tilde{\mu}_j^{(e)}$, j is in thermodynamic equilibrium across the plasma membrane,

$$\mu^O + RT \ln a_j^{(c)} + z_j F \psi^{(c)} = \mu^O + RT \ln a_j^{(e)} + z_j F \psi^{(e)}$$

which leads to:

$$\psi^{(c)} - \psi^{(e)} = \frac{RT}{z_j F} \ln \frac{a_j^{(e)}}{a_j^{(c)}}$$

At electrochemical equilibrium the potential difference, $\psi^{(c)} - \psi^{(e)}$, is denoted as j's *equilibrium potential*, E_j, at the prevailing ion activities,

$$E_j = \frac{RT}{z_j F} \ln \frac{a_j^{(e)}}{a_j^{(c)}} \approx \frac{RT}{z_j F} \ln \frac{c_j^e}{c_j^c} \quad (6)$$

Because the ionic strengths of the cellular and extracellular fluids are about the same, as a good approximation, $f_j^{(c)} \approx f_j^{(e)}$, whereby concentrations of j can replace the activities as indicated in Eq. (6), which is the *Nernst Equation*. Thus, given the concentrations, $c_j^{(e)}$ and $c_j^{(c)}$, E_j is the electrical potential difference required between the two compartments for j being in electrochemical equilibrium. Fig. (1B) shows an epithelium of polarized cells with tight junctions between the cells separating the extracellular fluid from the environmental or the luminal fluid. As indicated, there are several anatomical pathways for exchange of ions and water between the extracellular fluid and the fluid bathing the outside or luminal aspect of the epithelium. The above thermodynamic equilibrium condition applies as well to the ion distribution across an epithelium no matter which and how many of the pathways are involved in the transepithelial transport. For transepithelial transport, the symbols $\tilde{\mu}_j^{(c)}$, $f_j^{(c)}$, $a_j^{(c)}$, $c_j^{(c)}$ and $\psi^{(c)}$ are replaced by the symbols $\tilde{\mu}_j^{(o)}$, $f_j^{(o)}$, $a_j^{(o)}$, $c_j^{(o)}$ and $\psi^{(o)}$ with the new superscript, (o), referring to the outside (environmental or luminal) fluid.

Membrane potentials: The homogenous membrane

In a multi-ion system, membrane potential, $V_m = \psi^{(c)} - \psi^{(e)}$, depends on permeability and distribution of all membrane-permeable ions. While the Nernst equation for the equilibrium distribution of a single ion (Eq. 6) is independent of membrane structure, the mathematical equation for V_m for the general non-equilibrium case can only be derived for simplifying assumptions about the membrane's physical properties. The *Goldman potential equation* was derived for a *homogenous membrane* (see below) for the case V_m is a pure diffusion potential governed by the three major diffusible ions (775),

$$V_m = \frac{RT}{F} \ln \frac{P_{Na} c_{Na}^{(e)} + P_K c_K^{(e)} + P_{Cl} c_{Cl}^{(c)}}{P_{Na} c_{Na}^{(c)} + P_K c_K^{(c)} + P_{Cl} c_{Cl}^{(e)}} \quad (7a)$$

It is tacitly assumed that active Cl^- transport mechanisms are non-rheogenic, and that the contribution of the rheogenic Na^+/K^+ pump to the membrane potential can be neglected. A corollary to the last mentioned assumption is that the specific plasma membrane resistance, R_m, is low. Otherwise, the pump's contribution to the membrane potential, ΔV^{pump}, cannot be neglected, $\Delta V^{pump} = R_m I^{pump}$, where I^{pump} is the pump current. At *steady state* with a cation stoichiometry of the Na^+/K^+ pump of $\beta = 3/2$, i.e., $I^{pump} = F(\beta - 1) \cdot J_K^{pump}$, Eq. (7a) reads:

$$V_m = \frac{RT}{F} \ln \frac{P_{Na} c_{Na}^{(e)} + \beta P_K c_K^{(e)} + P_{Cl} c_{Cl}^{(c)}}{P_{Na} c_{Na}^{(c)} + \beta P_K c_K^{(c)} + P_{Cl} c_{Cl}^{(e)}} \quad (7b)$$

In the above equations, the permeability coefficient, P_j is defined as

$$P_j = \frac{\alpha_j D_j}{h} \quad (8)$$

where α_j is the dimensionless partition coefficient relating the ion's concentration in the solution to its concentration just inside the membrane boundary, D_j is the diffusion coefficient in the membrane, and h is the membrane thickness. If D_j is in $cm^2 \cdot s^{-1}$ and h is in cm, P_j is in $cm \cdot s^{-1}$. The homogenous membrane fulfils the following requirements: (i) D_j is independent of the position (x) in the membrane; (ii) the membrane is symmetric, which means that α_j at the intracellular membrane-fluid boundary ($x = 0$) is the same as α_j at extracellular membrane-fluid boundary ($x = h$); (iii) the electrical field in the membrane is constant, which means that the electrical potential is a linear function of x; and (iv) the potentials at $x = 0$ and $x = h$ are equal to the potentials in the cellular and extracellular fluids, respectively.

Transport Equations

Diffusion

Net transport by diffusion is a random thermal movement of molecules of a non-uniform distribution in a solution or between compartments. Thus, if there is a concentration difference across the membrane of a non-charged solute, there will be a net transport in direction from the compartment of higher concentration to that of lower concentration, which is governed by Fick's first law of diffusion in one dimension along the x-coordinate,

$$J_s = -D_s \frac{dc_s}{dx} \quad (9a)$$

The diffusion coefficient, D_s, and the concentration gradient, dc_s/dx, refer to the same arbitrary position along the transport path in the membrane. For a homogenous membrane, integration of Eq. (9a) leads to

$$J_s = P_s(c_s^{(c)} - c_s^{(e)}) \tag{9b}$$

The permeability coefficient, P_s, is related to the diffusion coefficient in the membrane by Eq. (8) above. Equation (9b) follows the convention that a flux directed from the cell to the extracellular fluid is given a positive sign. For a membrane composed of layers of different thicknesses and diffusion coefficients, which may include external unstirred layers, P in Eq. (9b) is replaced by an equivalent permeability, $\langle P \rangle$, which is given by the permeability of the individual layers according to $1/\langle P \rangle = 1/P_1 + 1/P_2 + \ldots\ 1/P_n$ (804, 1761).

Electrodiffusion

An electrical field, $d\psi/dx$, superimposed on the random movement of ions in the membrane expands Eq. (9a) to

$$J_j = -D_j \frac{dc_j}{dx} + D_j \frac{z_j F c_j}{RT} \frac{d\psi}{dx} \tag{10a}$$

For the *homogenous membrane*, integration of Eq. (10a) leads to the *Goldman flux equation* (Eq. 10b) and the *Goldman current equation* (Eq. 10c), respectively (775, 1761),

$$J_j = \left[P_j \frac{z_j F V_m}{RT} \right] \frac{c_j^{(e)} - c_j^{(c)} \exp\left\{z_j F V_m/(RT)\right\}}{1 - \exp\left\{z_j F V_m/(RT)\right\}} \tag{10b}$$

$$I_j = \left[P_j \frac{z_j^2 F^2 V_m}{RT} \right] \frac{c_j^{(e)} - c_j^{(c)} \exp\left\{z_j F V_m/(RT)\right\}}{1 - \exp\left\{z_j F V_m/(RT)\right\}} \tag{10c}$$

where, $I_j = z_j F J_j$, P_j is given by Eq. (8) and $V_m = \psi^{(c)} - \psi^{(e)}$. For $c_j^{(e)} \neq c_j^{(c)}$, the current-voltage relationship given by Eq. (10c) is non-linear, which is referred to as Goldman-Hodgkin-Katz rectification (GHK rectification). In characterizing the molecular phenotype of ion channels, Eq. (10c) is a useful reference. For example, the epithelial sodium channel (ENaC), which is expressed in many Na^+-absorbing epithelia, exhibits GHK rectification (562, 1389, 1390, 1899). Single-channel studies of another important epithelial ion channel, the CFTR chloride channel, indicated that under certain conditions, the channel exhibits a more complicated Cl^- current rectification than Eq. (10c) predicts (1069, 1796).

Convection superimposed on diffusion: Solvent drag

In fluid-transporting epithelia, water transport through water- and solute-permeable pores acts as a force in the direction of the water flow that drives the solute from one side of the pore to the other (27). With v being the convection velocity ($cm \cdot s^{-1}$) in the pore and D_s the diffusion coefficient of the solute in the pore, the extension of Eq. (9a) reads:

$$J_s = -D_s \frac{dc_s}{dx} + v c_s \tag{11a}$$

Integration through a homogenous pore of uniform cross-section area gives *Hertz's equation* (1761),

$$J_s = v \frac{c_s^{*(e)} - c_s^{*(c)} \exp(vh/D_s)}{1 - \exp(vh/D_s)} \tag{11b}$$

The symbols $c_s^{*(c)}$ and $c_s^{*(e)}$refer to concentrations *in* the pore at $x = 0$ and $x = h$, respectively. Considering 1 cm^2 of cross-sectional pore area, the convection velocity in $cm \cdot s^{-1}$ is a measure of the volume flow per cm^2 of uniform membrane pore, J_V (in $cm^3 \cdot cm^{-2} \cdot s^{-1}$). Introducing P by Eq. (8), the reflection coefficient, σ, and the relationship for a symmetrical pore, $\alpha = 1 - \sigma$ (532), Eq. (11b) becomes

$$J_s = [(1-\sigma)J_v] \frac{c_s^{(e)} - c_s^{(c)} \exp\{(1-\sigma)J_V/P_s\}}{1 - \exp\{(1-\sigma)J_V/P_s\}} \tag{11c}$$

For an ion that is transported under the influence of a water flow and an electrical field, as for example at tight junctions, Eq. (9a) is expanded to Eq. (11d), which integrates to Eq. (11e) below (1005):

$$J_j = -D_j \frac{dc_j}{dx} + D_j \frac{z_j F c_j}{RT} \frac{d\psi}{dx} + v c_s \tag{11d}$$

$$J_j = \left[P_j \frac{z_j F V_m}{RT} + (1-\sigma) J_v \right] \times \frac{c_j^{(e)} - c_j^{(c)} \exp\left\{z_j F V_m/(RT)\right\} \exp\left\{(1-\sigma)J_V/P_j\right\}}{1 - \exp\left\{z_j F V_m/(RT)\right\} \exp\left\{(1-\sigma)J_V/P_j\right\}} \tag{11e}$$

The flux-ratio equation

Equations 9-11 depend on the assumption that the transport pathway is *homogenous* as defined above, which is not fulfilled in multi-membrane systems like epithelia. If the transport across a barrier of arbitrary complexity, such as an epithelium, takes place entirely as a consequence of differences in concentration and electrical potential between the two sides of the barrier, and if j is not interacting with other moving particles, the *flux-ratio equation* is obeyed (1889):

$$RT \ln \frac{J_j^{(in)}}{J_j^{(out)}} = \tilde{\mu}_j^{(o)} - \tilde{\mu}_j^{(i)} \qquad J_j^{(net)} = J_j^{(in)} - J_j^{(out)} \tag{12a}$$

Insertion of Eqs. (5a) and (5b) into Eq. (12a) yields

$$RT \ln \frac{J_j^{(in)}}{J_j^{(out)}} = RT \ln \frac{a_j^{(o)}}{a_j^{(i)}} + z_j F(\psi^{(o)} - \psi^{(i)}) \quad (12b)$$

which can be rearranged to give

$$\frac{J_j^{(in)}}{J_j^{(out)}} = \frac{a_j^{(o)}}{a_j^{(e)}} \exp \frac{z_j F V_T}{RT} \quad (12c)$$

$$\frac{J_j^{(in)}}{J_j^{(out)}} = \exp \frac{z_j F(V_T - E_j)}{RT} \quad (12d)$$

where $V_T = \psi^{(o)} - \psi^{(e)}$ is the electrical potential difference across the epithelium, and E_j in Eq. (12d) is the equilibrium potential (Eq. 6). A straightforward way of proving this classical and important equation is presented in ref. (1761). Thus, a flux ratio analysis constitutes a powerful method of identifying the mechanism of transport of an ion in question. The influx and the efflux are measured by means of radioactive isotopes of the ion, or by isotope tracer technique for measuring one of the unidirectional fluxes combined with chemical determination of the net transport. Knowing the ion activities (or the ion concentrations if $f_j^{(o)} = f_j^{(i)}$) and the trans-epithelial potential difference, by means of Eq. (12d) it is revealed whether the transport is simple passive (954).

Active Transport: Free Energy Change of ATP Hydrolysis

Active transport occurs when the flux is coupled with an exergonic process at the plasma membrane, which may be hydrolysis of ATP (*primary active transport*) or a passive flux of another ion (*secondary active transport*) transported either in the same direction of *j* (*cotransport*) or in the opposite direction of *j* (*counter transport*). The hydrolysis of ATP proceeds as follows,

$$ATP^{4-} + H_2O \rightarrow ADP^{3-} + HPO_4^{2-} + H^+,$$

with the change in free energy associated with the hydrolysis of 1 mol ATP given by

$$\Delta G_{ATP^{4-}}^{(c)} = \Delta G^{o'} + RT \ln \frac{c_{ADP^{3-}}^{(c)} c_{HPO_4^{2-}}^{(c)}}{c_{ATP^{4-}}^{(c)}}, \text{pH}^{(c)} = 7.0$$

where $\Delta G^{o'} = \Delta G^{o} + RT \ln c_{H^+}^{(c)} = -30.5 \text{ kJ} \cdot \text{mol}^{-1}$ is the change in standard free energy at 25 °C at 1 M concentration of reactants and products, at $c_{H^+}^{(c)} = 10^{-7}$ M. At typical cellular concentrations, $\Delta G_{ATP^{4-}}^{(c)} \approx -60 \text{ kJ} \cdot \text{mol}^{-1}$. This would be the theoretical upper-limit electrochemical work of an ion pump energized by ATP hydrolysis, noting that 100% energetic coupling efficiency cannot be achieved.

Thermodynamic work of ion pumps

In the context of osmoregulation of freshwater animals, the thermodynamic work associated with branchial or integumental transcellular uptake of Na^+ and Cl^- is of particular interest. These osmoregulatory and respiratory tissues express the P-type Na^+/K^+ pump ATPase at the (baso)lateral plasma membrane (858, 1257, 1275, 1545, 1709, 1710) and the V-type H^+ pump ATPase at the apical plasma membrane (922, 945, 1058, 1062, 1303, 1412, 1848, 1949). At physiological conditions one cycle of the Na^+/K^+ pump results in translocation of 3 Na^+ from the cellular to the extracellular fluid and 2 K^+ in the opposite direction at the expense of hydrolysis of 1 ATP^{4-} to 1 ADP^{3-} and 1 HPO_4^{2-},

$$3Na^{+(c)} + 2K^{+(e)} + ATP^{4-} + H_2O^{(c)} \rightarrow 3Na^{+(e)} + 2K^{+(c)} + ADP^{3-} + HPO_4^{2-} + H^+$$

In osmoregulatory epithelia, the 3 Na^+ are taken up across the apical plasma membrane, while the 2 K^+ recycle across the basolateral plasma membrane (956, 1312). Thus, the theoretical maximum work that can be done in transporting 1 mol of Na^+ across the epithelium is $-\Delta G_{ATP^{4-}}^{(c)}/3 \approx 60/3 = 20$ kJ. A single cycle of the H^+ pump ATPase leads to secretion of 2 H^+ to the outside fluid at the expense of the hydrolysis of 1 ATP^{4-} together with the exchange of 2 Cl^- in the outside bath with 2 HCO_3^- in the cellular fluid,

$$2H^{+(c)} + 2HCO_3^{-(c)} + 2Cl^{-(o)} + ATP^{4-(c)} + H_2O^{(c)} \rightarrow 2H^{+(o)} + 2HCO_3^{-(o)} + 2Cl^{-(c)} + ADP^{3-(c)} + HPO_4^{2-(c)} + H^{+(c)}$$

Subsequently, 2 Cl^- are passively transported across the basolateral membrane to the extracellular fluid. In this case, the theoretical maximum work that can be done in transporting 1 mol Cl^- across the epithelium is $-\Delta G_{ATP^{4-}}^{(c)}/2 \approx 60/2 = 30$ kJ. With reference to Eqs. (5a) and (5b) above, and with superscript (*o*) for freshwater, the electrochemical work done in transporting 1 mol of Na^+ and 1 mol of Cl^- from freshwater to the extracellular fluid as a first approximation is

$$\begin{aligned} \Delta\tilde{\mu}_{Na} + \Delta\tilde{\mu}_{Cl} &= RT \ln \frac{a_{Na}^{(e)}}{a_{Na}^{(o)}} + FV_T \\ &\quad + RT \ln \frac{a_{Cl}^{(e)}}{a_{Cl}^{(o)}} - FV_T \\ &= RT \ln \frac{f_{Na}^{(e)} c_{Na}^{(e)}}{f_{Na}^{(o)} c_{Na}^{(o)}} + RT \ln \frac{f_{Cl}^{(e)} c_{Cl}^{(e)}}{f_{Cl}^{(o)} c_{Cl}^{(o)}} \end{aligned} \quad (13)$$

Energy expenditure of ion uptake from diluted solutions

Assume extracellular concentrations of Na^+ and Cl^- of 125 mM, and a ratio of activity coefficients of 0.78; if only the $3Na^+/2K^+$ pump ATPase is involved and the Cl^- uptake is

passive and driven by the transepithelial potential, the uptake of the two ions would cease already at an external concentration of Na^+ and Cl^- between 5 and 1 mM ($\Delta\tilde{\mu}_{Na} + \Delta\tilde{\mu}_{Cl} \approx$ 15 and 23 kJ, respectively). This is well above the ion concentrations of a majority of freshwater pools. With the additional contribution of the H^+ ATPase (846, 848, 1371, 1379, 1424, 1425), whereby $-(\Delta G^{(c)}_{ATP^{4-}}/3 + \Delta G^{(c)}_{ATP^{4-}}/2) \approx 50$ kJ, the uptake of 1 mol Na^+ and 1 mol Cl^- would be possible at about 10 μM ($\Delta\tilde{\mu}_{Na} + \Delta\tilde{\mu}_{Cl} \approx 45$ kJ). In his study published in 1937, Krogh observed that Cl^- is taken up by a salt-depleted frog immersed in solutions down to about 10 μM provided Na^+ constituted the matching cation (978). If the apical anion exchanger is rheogenic with a stoichiometry of $Cl^-/nHCO_3^-$ ($n > 1$) and still coupled to an apical H^+ ATPase, it would be possible to take up Na^+ and Cl^- at even more diluted environments (657). For example, if $n = 2$, two protons are pumped out of the cell for each Cl^- taken up via the anion exchanger thus increasing the ATP: Cl^- stoichiometry from 0.5 to 1. Therefore, the theoretical maximum work in transporting 1 mol of Na^+ and 1 mol Cl^- becomes 80 kJ rather than 50 kJ, which would make transepithelial uptake of the two ions thermodynamically possible at environmental concentrations of Na^+ and Cl^- in the order of 100 nM.

The above examples illustrate principles of energy transfer in active ion transport. In a given situation, temperature and concentrations of the participating ions and metabolites have to be considered. If energetically feasible, whether uptake of Na^+ and Cl^- does take place depends on the affinity of the ion-binding sites of the transport systems. The zebrafish (*Danio rerio*) has the capacity for increasing the affinity of its branchial transport systems upon acclimation to micromolar Na^+ and Cl^- concentrations (150), which indicates that this species would be able to exploit the power of the above combination of transport ATPases.

Solute-Coupled Water Transport and Isotonic Transport

Early studies of water transport by kidney proximal tubule (1618), small intestine (364) and gallbladder (423) revealed a sizable transepithelial fluid flow at transepithelial osmotic equilibrium of an osmolality similar to that of the bathing solutions. The rate of fluid transport was proportional with the active flux of Na^+. A similar "isotonic transport" has been observed for the acinar epithelium of exocrine glands (1819, 1820), Malpighian tubules (1998) and amphibian skin epithelium (1313, 1314). For fluid-absorbing epithelia it was suggested that such a flow of water in the absence of a transepithelial osmotic pressure difference proceeds in two steps (363, 1982). Apically, transport of water into the lateral space is forced by a small osmotic pressure difference between the luminal (external) solution and the lateral intercellular space. Subsequently, water exits across the interspace basement membrane of a reflection coefficient near zero, thus being forced by the hydrostatic pressure difference between the lateral space and the interstitial space (conf. Eq. 4a). In entering the epithelium, water may take a transjunctional and/or a translateral route into the lateral space (defined in Fig. 1B), with additional solute-water couplings in the lumen of basolateral infoldings (1866). This hypothesis is supported by the finding that sodium pumps in transporting epithelia are expressed preferentially or exclusively at the lateral membranes and at the membranes of basolateral infoldings (499, 658, 886, 1228, 1229, 1457, 1770). With no further assumptions, in principle this hypothesis accounts for uphill water transport as well (998, 1961). It is not immediately obvious, however, how the transported fluid becomes isotonic at transepithelial equilibrium, because the fluid flowing out of the lateral space through the interspace basement membrane would be hypertonic. Careful experimental analysis has shown that the osmotic water permeability of some but not all transporting epithelia is very high, resulting in a tonicity of the transported fluid that was estimated to be only slightly hypertonic. Thus, it was suggested to replace the concept of isotonic transport with that of "nearly isotonic transport" (1747).

Theories of truly isotonic transport

Theories have been developed that handle "truly isotonic transport." In the Diamond-Bossert *standing gradient theory* (424), it was assumed that tight junctions are water impermeable and sodium pumps are concentrated at the lateral membranes near the luminal end, so this region of the lateral space becomes sufficiently hypertonic for driving water into the space from the luminal solution via cells. Next, the osmotic pressure gradient is being dissipated as water flows into the lateral space from the cells (see Fig. 2A). Mathematical analysis (424) showed that by appropriate combination of the transport variables osmotic equilibrium can be achieved at the boundary between the lateral space and the interstitial space. Diamond and Bossert rationalized their theory by pointing out that diffusion of solutes out of the lateral space would be very large as compared to the convection term even in the presence of a small difference in solute concentration between the lateral fluid and the interstitium, which would result in a strongly hypertonic fluid emerging from the lateral space (424). This pertinent feature of a convection-diffusion process at a membrane of high solute diffusion permeability is recognized by function analysis of Eq. (11c) (997, 1006).

In the *Na^+ recirculation theory* (Ussing), it is acknowledged that the fluid emerging from osmotic coupling compartments is hypertonic and assumed that simultaneous stationary fluxes return Na^+ (and Cl^-) into the coupling compartments from the interstitial space via the cells (1005, 1009, 1301) (see Fig. 2B). According to this theory, isotonic transport is achieved by a regulated balance between a forward flow of ions and water from the lateral space and the lumen of basal infoldings, respectively, into the interstitial fluid, and a backward flow of ions through the cells into the two types of coupling compartments (Fig. 2B), denoted by Ussing as "ion recirculation." At steady state, the entrance of water across the apical barriers is driven by the osmotic pressure difference

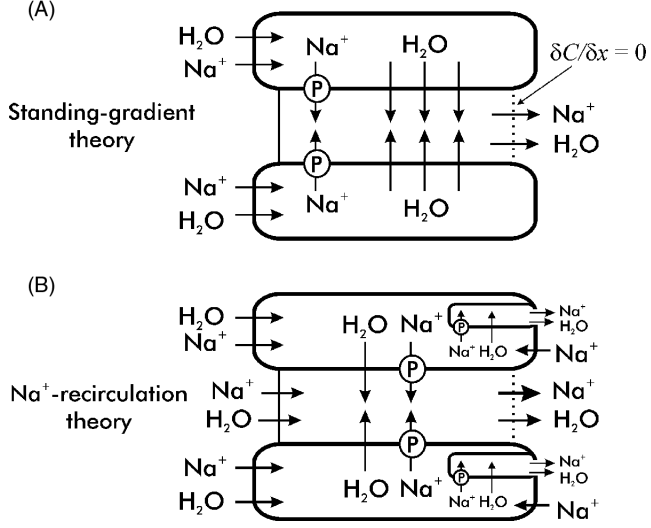

Figure 2 Theories of truly isotonic transport where all of the water exits the epithelium through the interface between the lateral intercellular and the interstitial space and through the opening of the basal plasma membrane infoldings as explained in the the text. (A) In the standing-gradient theory it is assumed that the lateral Na^+/K^+ pumps are displayed near the tight junction and the water channels in the lateral plasma membrane along the entire lateral intercellular space. With appropriate choice of model variables, the condition of zero solute concentration gradient at the interface between the lateral intercellular space and serosal bath is obtained (424). (B) The Na^+ recirculation theory. The boundary condition of Fig. 2A is replaced by the assumption that a regulated back flux of ions through the serosal plasma membrane, driven by the Na^+/K^+ pumps, adjusts the composition of the absorbed fluid to the demanded isotonicity (1301). Note that the Na^+/K^+ pumps play a dual role for isotonic transport. Firstly, the pump activity maintains the driving force for water uptake from the luminal solution. Secondly, the pump activity energizes the ion recirculation for achieving an isotonic net transportate. Modified from (1009).

between the osmotic coupling compartments and the luminal solution. Thus, at constant pump fluxes (and volume flow), the osmotic pressure of the lateral space and the lumen of basal plasma membrane infoldings, respectively, increases with decreasing hydraulic conductance of the apical barriers, resulting in an increased diffusion flux out of the osmotic coupling compartments into the interstitial fluid. It follows that the demand of solute recirculation for achieving overall isotonic transport depends critically on the hydraulic conductance of the apical barriers (998, 1006). For comparison, the assumptions of the standing gradient theory and the Na^+ recirculation theory, respectively, are outlined in parallel panels of Fig. 2. For both theories of passive inflow of water across the apical cell membrane, it is important to realize that under the condition of osmotic equilibrium between the luminal (apical) and the interstitial (serosal) space, water is entering the epithelial cells also across their basal membrane, if this membrane is water permeable. It follows that all of the water exits the epithelium through the interface between the lateral intercellular space and the basal infoldings, respectively, and the interstitial space, the mechanism of which is in the focus of the Na^+ recirculation theory.

Quite different theories of driving forces for isotonic fluid transport are also discussed in the literature. Firstly, experimental (1599) and theoretical (1584) studies have led to the suggestion that *electro-osmotic coupling at tight junctions* represents one of the basic mechanisms driving fluid transport across some leaky epithelia (534). According to this theory, secretion of a paracellular fluid flow is driven by a Na^+ current in leaky tight junctions lined by fixed negative charges. The paracellular Na^+ current is assumed energized by a lumen-negative transepithelial potential generated by active transcellular ion fluxes (535). Two more theories of isotonic transport are water transport by a Na^+/glucose transporter (SGLT) in the luminal plasma membrane (1218), and active ATP hydrolysis-driven water transport at tight junctions (750). With emphasis on experimental support and short comings, the main ideas of each of the above five theories of isotonic transport are discussed in refs. (998, 1009).

Annelida and Mollusca

Molluscs and annelids inhabit a wide variety of habitats, including the ocean, estuaries and oligohaline seas, freshwater lakes and rivers, hypersaline waters, and land. Each habitat presents the animals living in it with a different mix of environmental variables; evolution has produced a broad diversity of physiological mechanisms involved in salt and water balance in these groups of animals. The salinity of the water is an important environmental factor that determines the diversity of living organisms in aquatic habitats. This relationship is illustrated by the classic curve of Remane (1520) (Fig. 3).

Species diversity is very high in marine and freshwaters, decreases markedly in brackish waters, and is quite low in hypersaline habitats. Clearly, the physiology of salt and water balance is a highly significant factor in the evolution of diversity among aquatic animals. Many marine invertebrates are osmotic conformers—that is, the osmotic concentration (and usually the ionic composition) of the extracellular fluid is similar to that of the ambient seawater. In contrast, all freshwater species are osmotic hyper-regulators, with an

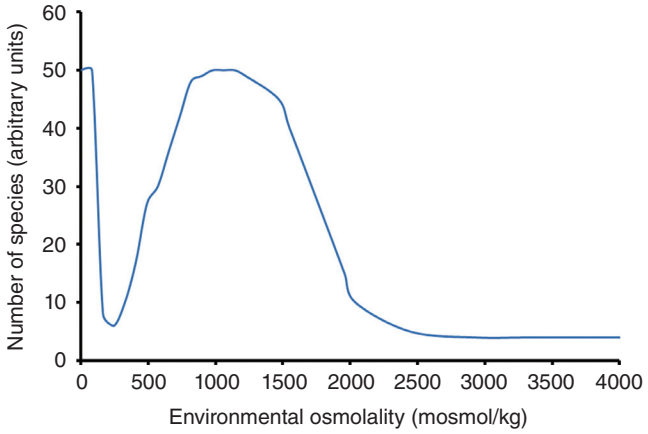

Figure 3 Number of aquatic species in relation to environmental salinity. Redrawn from Remane (1520).

extracellular fluid osmotic concentration higher than that of the ambient medium. Most animals that inhabit brackish water show a combination of these behaviors: the extracellular fluid conforms to the ambient medium at higher salinities and is hyperosmotic to the medium at lower salinities. For animals in terrestrial habitats, the primary factor in the maintenance of salt and water balance is evaporative water loss to the environment. These animals obtain water by drinking and from their food. Ions are obtained from the diet.

Marine Osmoconformers

Marine animals that are osmotic conformers may be either stenohaline (able to survive within a narrow range of environmental salinity) or euryhaline (tolerant of a wide range of salinity). In these animals, the osmotic concentration of the body fluids is usually slightly hyperosmotic to the ambient concentration (see [1359, 1681] for examples). The ionic composition of the hemolymph of osmotic conformers is, with a few exceptions, nearly identical to the ambient seawater (Table 1). Pelagic animals have reduced concentrations of sulfate in the body fluids, probably to increase buoyancy. The urine produced by these animals is iso-osmotic to the medium (Table 2). Because of wide differences in methodology, the values for urine production rates in Table 2 should be accepted with caution. Nonetheless, the rates of urine production in some of marine animals are not markedly different from those found in comparable freshwater animals; the reasons for this are unknown. Stenohaline bivalves are more permeable to water than are intertidal species, but the permeability of freshwater clams is as high as that of marine species (1491). An osmotic conformer exposed to a large decrease in the osmotic concentration of the ambient medium will take up water by osmosis and lose ions by diffusion. The magnitude of these diffusive fluxes depends on the scale of the change in the ambient osmolality. Reductions in the permeability of the body wall to water have been reported in both molluscs (406, 1491) and polychaetes (539) exposed to reduced osmotic concentrations. This mechanism would slow the rate of water influx and provide the animal with more time to compensate. The reported decreases in permeability are, however, small in comparison to differences in the permeability of the body wall between marine and freshwater animals (941).

Marine osmotic conformers: Volume regulation

For an osmoconformer, any change in the ambient osmotic concentration results in an osmotic gradient between the extracellular fluids (and cells) and the surrounding medium. The process that returns the cytoplasmic and extracellular fluid compartments to osmotic equilibrium with the ambient medium is called volume regulation. If the ambient salinity increases, the animal loses water; a decrease in the ambient salinity results in an uptake of water. In many of these animals, the initial gains or losses in water are partially or

Table 1 Osmotic (π, mosmol/kg) and Ionic (mM) Concentrations in Seawater and the Body Fluids of Soft-Bodied Invertebrate Osmotic Conformers

	π	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	Reference
Seawater	**1,033**	**480**	**10**	**10**	**55**	**560**	**29**	**(74)**
Species		**Internal Ratio/External**						
Aurelia aurita (C)	0.999	0.989	1.058	0.956	0.972	1.038	0.471	(1564)
Phascolosoma vulgare (S)	—	1.038	1.057	1.043	0.686	0.985	0.913	(1563)
Aphrodita aculeata (P)	—	0.994	1.260	1.000	0.988	1.003	0.999	(1359)
Arenicola marina (P)	—	1.001	1.035	0.998	1.003	0.997	0.992	(1359)
Amphitrite brunnea (P)	—	0.976	1.429	1.016	1.100	0.988	1.027	(1359)
Mercierella enigmatica (P)	1.140	0.903	1.670	0.882	1.238	0.941	—	(1359)
Mytilus edulis (B)	—	0.990	1.183	1.135	0.965	0.989	1.004	(1474)
Pecten maximus (B)	—	0.997	1.325	1.017	0.784	0.976	1.029	(717)
Strombus gigas (G)	—	0.998	1.080	1.068	1.074	1.007	0.717	(1079)
Scutus breviculus (G)	1.012	1.008	1.036	1.033	1.025	—	0.987	(1875)
Eledone cirrosa (Cp)	—	0.986	1.540	1.167	1.084	1.008	0.780	(1567)
Sepia officianalis (Cp)	—	0.889	0.991	1.075	1.090	1.085	—	(1)
Octopus dofleini (Cp)	—	0.911	1.132	0.921	—	0.941	0.735	(1477)

C = Colenterate, S = Sipunculid, P = Polychaete, B = Bivalve, G = Gastropod, Cp = Cephalopod

Table 2 Urine Production in Soft-Bodied Invertebrates

Species	π_{ext} (mosmol/kg)	Urine/hemolymph ratio	Rate (μl $g^{-1}hr^{-1}$)	Reference
Strombus gigas (G)	1,100	0.96	3	(1079)
Haliotis rufescens (G)	1,100	1.0	6-20	(701)
Octopus dofleini (Cp)	1,100	1.0	3	(702)
Nereis diversicolor (P)	1,100	1.0	3	(1720, 1721)
Anodonta cygnea (B)	FW	0.67	12	(1446, 1475)
Margaratana margarantifera (B)	FW	0.09	3-15	(267)
Pomatia limata (G)	FW	0.23	6	(1077)
Viviparus viviparus (G)	FW	0.28	15	(1078)
Hirudo medicinalis (H)	FW	0.10	40	(1965)
Nereis diversicolor (P)	70	0.60	30-130	(1721, 1722)
Lumbricus terrestris (O)	FW	0.19	7	(430, 1504)
Helix pomatia (G)	T	—	10	(1916)
Achatina fulica (G)	T	0.72	4	(1172)

G = Gastropod, Cp = Cephalopod, P = Polychaete, B = Bivalve, H = Hirudinea, O = Oligochaete

completely reversed. The capacity for volume regulation in marine osmotic conformers varies from extremely limited (e.g., sipunculids) to very high (some polychaetes and molluscs) (467, 574, 1360, 1447, 1914). Transfer of the sipunculid *Themiste dyscritum* from seawater to 50% seawater results in a 65% increase in the weight of the animal; there is no volume regulation (1360). The polychaete *Arenicola marina* is able to volume regulate completely when transferred from 32‰ to 20‰ but retains about 10% of the water gained when the transfer is to 10‰ (1519). The bivalve *Geukensia demissa* regains volume in 48 hr following a transfer from 36‰ to 3‰ (1447). There is no evidence of volume regulation in *G. demissa* transferred from 36‰ to 48‰ (1447). In both molluscs and annelids, the responses to hypo- and hyperosmotic media are asymmetrical: volume regulation is more rapid and more complete following exposure to hypoosmotic media than following exposure to hyperosmotic media (573, 574, 1362).

The cells of these animals respond to osmotic swelling or shrinkage by adjustment of the cytoplasmic pool of osmolytes. The primary inorganic ion in the intracellular osmotic pool is K^+, but the concentration of potassium ions in the cytoplasm is always below about 200 mM (941). Since the osmotic concentration of the body fluids (and therefore, the cells) of an animal that is a conformer at higher salinities can be over 2000 mosmol/kg (1450), the cytoplasm of marine animals contains high concentrations of organic molecules such as amino acids and quaternary amines. High concentrations of these compatible solutes are less deleterious to protein structure and function than high concentrations of inorganic ions (2050). The amino acids that constitute the cytoplasmic pool vary among species and even among populations of the same species (958, 1449, 1939). The amino acids that are commonly abundant include taurine, alanine and glycine; β-alanine is also abundant in coelenterates (408, 467, 887, 958, 1519). Quaternary amines include proline betaine and glycine betaine (1448, 1449).

In animals exposed to a decrease in salinity, the osmotic concentration of the extracellular fluid falls, and the cells take up water. The cells release cytoplasmic ions and organic osmolytes into the extracellular fluid to eliminate the osmotic gradient across the plasma membrane (408, 554). The amino acids released by the cells are not excreted; most are deaminated by unknown tissues in the animal; the ammonia is excreted, but the carbon skeletons are presumably conserved (75, 1149). Exposure of animals to an increase in the ambient salinity results in loss of water from their cells. Accumulation of cytoplasmic osmolytes reverses this osmotic loss of water in those species capable of volume regulation. In bivalve molluscs, the cytoplasmic levels of alanine rise very quickly following transfer to a hyperosmotic medium; over time, glycine and taurine accumulate more slowly (60). The complex changes in amino acid metabolism that are involved in hyperosmotic volume regulation in bivalves have been studied (142).

The efficacy of volume regulatory mechanisms in excitable cells in a few euryhaline invertebrates has been studied in detail. There is variability in the responses of the neurons of annelids and bivalves, but these cells adjust rather rapidly to dilute media and can generate action potentials despite large decreases in the intracellular concentrations of Na^+ and K^+ (90, 110, 1999). The isolated ventricles of bivalve molluscs also recover rapidly from either dilution or concentration of the bathing medium and initiate spontaneous mechanical activity within a few hours of the change in the ambient osmotic concentration (1937).

Table 3 Aspects of Osmoregulation in Oligohaline and Freshwater Soft-Bodied Invertebrates

	π_{ECF} in FW mosmol/kg	π_{break} mosmol/kg	Reference
Oligohaline Animals			
Polymesoda caroliniana (B)	48	60	(407)
Mytilopsis leucophaeta (B)	40	70	(410)
Potamopyrgus jenkinsi (G)	125	125	(460)
Assiminea grayana (G)	180	150	(1080)
Enchytraeus albidus (O)	404	400	(593)
Nereis diversicolor (P)	180	335	(1720)
Nereis limnicola (P)	175	350	(1361)
Freshwater Animals			
Limnoperna fortunei (B)	40	70	(410)
Lampsilis teres (B)	50	50	(855)
Lymnaea stagnalis (G)	95	100	(404)
Pomacea bridgesi (G)	100	100	(855)
Craspedacusta sowberyi (C)	30	30	(537)
Lumbricus terrestris (O)	145	200	(252)

Animals are osmotic conformers in ambient concentrations above and hyper-regulators below π_{break}.
B = Bivalve, C = Coelenterate, G = Gastropod, O = Oligochaete

Oligohaline and Freshwater Animals

Oligohaline invertebrates that inhabit brackish waters are, for the most part, osmotic conformers at higher salinities and hyperosmotic regulators at low salinity. This also applies to most freshwater species; freshwater molluscs and annelids have a lesser tolerance for increased ambient salinity than do brackish water species (408, 1359). The external salinity that causes the onset of hyper-regulation of the extracellular fluid varies considerably among these animals (Table 3). In some animals the permeability of the body wall to ions is reduced when the animals are regulating the osmotic concentration of the body fluids (440, 1358). The osmotic concentration of the hemolymph of freshwater bivalves and coelenterates is lower than that of freshwater snails and annelids. This reduces the gradients for passive loss of ions to the medium and uptake of water by osmosis, but is not reflected in lower rates of urine production in freshwater bivalves (Table 2). All freshwater animals produce urine that is hypoosmotic to the body fluids; however, the ratio of the osmotic concentration of the urine to that of the hemolymph is lower in freshwater vertebrates than in freshwater molluscs and annelids (Table 2) (941). To compensate for diffusive losses of ions and ions lost in the urine, hyper-regulators must take up ions from the medium by active transport. In bivalves, the site of uptake of chloride and sodium is probably the gills; putative transporting cells have been described in at least one species (431, 907). Worms take up Na^+ and Cl^- ions across the body wall (20, 427). The K_M's of these ion uptake systems in molluscs and the earthworm are similar, ranging from 0.05 to 1.5 mM (408, 427, 430). The maximal rates of uptake are also similar in the earthworm (1 μmol/g dry wt/hr) to the lower end of the range of rates for freshwater molluscs (1-30 μmol/g/hr). The rates of uptake of Na^+ and Cl^- by the horse leech *Haemopis sanguisuga* in fresh water are about 5 μmol/g dry wt/ hr (976). These values are consistent with those reported for other freshwater animals. As in other animals, the uptake mechanisms for Cl^- and Na^+ are independent (306, 404, 429, 430, 976). There is a paucity of specific information on the identity of the proteins involved in the uptake of ions in these animals. The uptake of sodium by freshwater mussels involves a Na^+-H^+ exchanger (428). Chloride uptake in mussels is inhibited by 4,4′-Diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS), suggesting that a chloride exchanger participates in the process (431). In leeches, integumental uptake of sodium is inhibited by furosemide and amiloride; this suggests the involvement of a passive sodium channel and a Na^+-K^+-$2Cl^-$ exchanger (306). In freshwater bivalves, the concentration of bicarbonate in the hemolymph is roughly equal to that of chloride; in gastropods and annelids, chloride is the predominant anion (408, 1359). In some leeches, organic acid concentrations in the hemolymph can total 25 mM (1307).

Terrestrial Animals

For a terrestrial animal with an extracellular fluid osmotic concentration of 200-400 mosmol/kg, an ambient relative humidity of less than 99.5% results in evaporative loss of water. Terrestrial annelids and molluscs dehydrate rapidly in

Table 4 Evaporative Water Loss Rates in Terrestrial Soft-Bodied Invertebrates

Species	Air Relative Humidity (%)	Water Loss (μl/g/hr)	Reference
Lumbricus terrestris (O)	70-80	24	(251)
Limax maximus (G)	70-80	40	(1486)
Arion ater (G)	40-80	10	(807)
Helix aspersa (G)	55	45	(1120)
Cepaea nemoralis (G)	65	4	(244)

O = Oligochaete, G = Gastropod

dry air (Table 4). Terrestrial molluscs and annelids can survive the loss of 70%-90% of their total body water (1120, 1485, 1576). Water loss in gastropods without a shell (e.g., *Limax*) is not higher than that of shelled species (e.g., *Helix*). Snails can reduce evaporative water loss by two orders of magnitude by withdrawing into the shell; sealing the shell aperture with an epiphragm results in a further decrease of about 50% (1120). During prolonged exposure to desiccation, annelids reduce water loss by estivation inside a secreted mucus cocoon (475). Dehydrated terrestrial molluscs and annelids take up water by osmosis across the body wall when conditions permit (430, 1485). In terrestrial molluscs subjected to desiccation, the concentrations of organic and inorganic osmolytes in the cells increase as the osmotic concentration of the extracellular fluids rises (433, 1994).

Urine Formation

In molluscs, the initial stage of urine formation is filtration of the hemolymph. This occurs in the heart-pericardial organ complex. In many species, the hydrostatic pressure that drives filtration is generated by contractions of the heart that drive hemolymph into the pericardial cavity (540, 745). Podocytes have been described in tissues such as the auricle, pericardial gland and kidney in a wide variety of species (44, 45, 714, 1219, 1368, 1622, 1936). The podocytes and associated basal lamina form a selective filtration barrier; filtered hemolymph passes from the hemocoel into the pericardial cavity. The filtrate then flows through a short ciliated reno-pericardial canal to the kidney. The filtrate is processed as it passes through the kidney; in freshwater animals, ions are reabsorbed to produce hypoosmotic urine. There is some evidence that some of the cells in the kidney have a secretory function (918).

In annelids, the excretory organs are nephridia and the primitive condition is one pair of nephridia per segment. In many species, the number of nephridia is greatly reduced and may be limited to a single pair (e.g., fanworms). Podocytes have been found in association with lateral blood vessels adjacent to the nephrostome; presumably, filtration of the hemolymph occurs here (527, 837, 1719). The proximal end of the nephridium (nephrostome) opens to the coelomic cavity and the distal end opens to the outside of the animal. Processing of the urine occurs as it passes along the nephridial tubule from nephrostome to the excretory pore. In some leeches, the nephridia produce urine by secretion rather than filtration; this is an adaptation that facilitates the rapid excretion of the large salt and water loads the animals incur by feeding on body fluids (1965).

Biomineralization

Many species of molluscs produce a calcareous shell and the serpulid polychaetes surround themselves with calcareous tubes. In marine osmotic conformers, the concentration of calcium in seawater is the same (10 mM) as that of the hemolymph. In serpulid worms, formation of the tube requires the deposition of calcium carbonate into a solution (seawater) saturated with these ions. A gland in the anterior end of the animal produces calcareous granules suspended in a matrix that are secreted to the exterior; this material is molded into shape and then solidifies to form the tube (1694). Marine molluscs secrete calcium carbonate to form the shell. In freshwater habitats, calcium concentrations vary between 0.07 and 1 mM depending on the hardness of the water (878). The concentrations of Ca^{2+} in the hemolymph of freshwater molluscs range between 2 and 10 mM (408); shell-bearing molluscs that inhabit fresh waters must therefore accumulate large amounts of calcium against a concentration gradient. In molluscs, the tissue that secretes the shell is the mantle epithelium. The mechanism responsible for the translocation of calcium from the hemolymph to the extrapallial fluid is unknown, but the electrophysiology and the transport of ions in this tissue suggest that it does not actively transport Ca^{2+} from the hemolymph to the extrapallial fluid that bathes the shell (327, 811, 834). These results suggest that calcium ions are actively transported from the medium into the hemolymph by active transport at the gill. Measurements of Ca^{2+} uptake by intact freshwater clams and snails produce K_M values for uptake that range from 0.1 mM to 0.3 mM; the K_M for marine animals is 7.5 mM (408, 875).

Osmoregulation: Neural and Endocrine Control

The uptake of ions in some freshwater bivalves follows a diurnal rhythm (638, 1200). These observations suggest that the nervous system regulates the function of the uptake mechanisms. Serotonin and cAMP stimulate the uptake of ions

and the uptake of sodium ions is inhibited by prostaglandins in freshwater mussels (434, 638, 802, 1619). Neurosecretory products alter kidney function in freshwater snails (919). In leeches, the neuropeptides angiotensin II amide and FMRFamide affect the movement of sodium across the body wall and control resorption of ions by the kidney (1223, 1965). Volume regulation in marine molluscs is affected by neuropeptides including FMRFamide (409, 1963).

Crustacea

The Crustacea are a subphylum of the arthropods with approximately 50,000 described species. Crustaceans are predominantly aquatic animals inhabiting seawater, freshwater and all types of brackish waters. Many species are remarkably euryhaline, acclimating to extremely different environmental salinities. Members of some crustacean groups, including crabs, hermit crabs and woodlice, have adapted to a terrestrial life. Osmotic and ionic regulation in Crustacea has been studied on all levels, from the whole animal to single molecules, and results have been reviewed on a regular basis. This summary tries to address all major topics of crustacean osmotic and ionic regulation. For further details, the reader is referred to the following list of reviews, book chapters and books that address osmotic and ionic regulation in Crustacea or special topics of this area (556, 613, 740, 933-935, 941, 1085-1087, 1110, 1151, 1338, 1381, 1418, 1421, 1476, 1811, 1845, 1846, 1848).

Osmotic and Ionic Concentrations in the Hemolymph

Until the mid-20th century, studies of osmotic and ionic regulation in Crustacea were almost exclusively performed on whole animals, and even afterward a considerable amount of important information was collected in this way. The hemolymph osmolality and ion composition of many crustaceans were determined, and often their changes in response to exposure to different media were monitored. From this wealth of data (probably most extensively reviewed in [1151]) a number of conclusions are evident. Groups with clearly different osmoregulatory capabilities can be distinguished among the Crustacea (see Fig. 4).

Florkin (541) distinguished two types of osmotic regulation. Intracellular isosmotic regulation occurs in euryhaline crustaceans that did not evolve the capability to maintain osmotic gradients across their body surface. The osmoregulatory processes in this group of animals are limited to the adjustment of the osmotic concentration of all cells to the osmolality of the hemolymph, which is equal to the osmotic strength of the ambient medium. In contrast, extracellular anisoosmotic regulation occurs in those crustaceans that maintain an osmotic gradient across the body surface by active transport of ions (mainly NaCl) across epithelial tissues. The first group consists of osmoconformers, not maintaining considerable osmotic gradients across their body

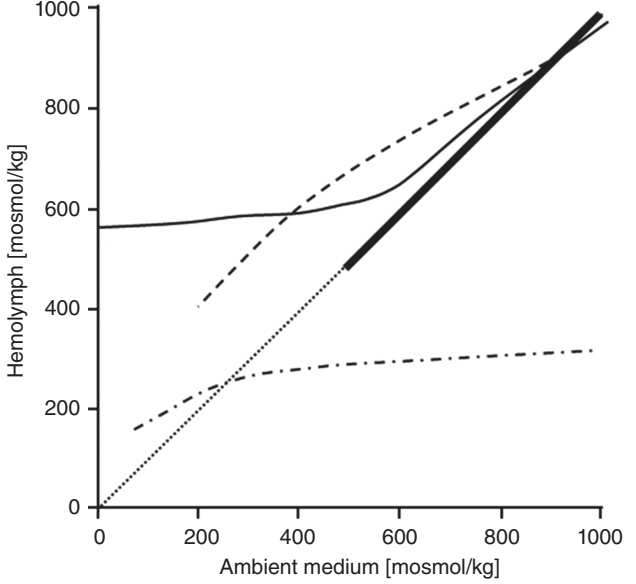

Figure 4 Osmotic concentration in the hemolymph of different crustaceans in ambient media of different osmotic strength. Isosmotic line (dotted line), osmoconformers (bold continuous line), weak hyperosmoregulator (dashed line; values for *Carcinus maenas*, recalculated after (1660), strong hyperosmoregulators (continuous line; values for *Eriocheir sinensis*, after [1372]), hyper-hyposmotic regulator (dotted and dashed line; *Artemia salina*, values after [360]).

surface. Thus, these animals do not spend energy for extracellular osmotic regulation. Although many of these animals inhabit marine environments, they can be considerably euryhaline. Migrating between seawater and brackish waters, their hemolymph osmolality changes considerably. In response to these changes euryhaline osmoconformers regulate the osmotic concentrations of the body cells, a process termed intracellular osmoregulation (see below). A considerable number of Crustacea are hyperosmoregulators, maintaining a higher osmotic concentration in their body fluids than in the aquatic environment. All freshwater species and euryhaline crustaceans that succeed to invade considerably dilute brackish waters and freshwater are hyperosmoregulators. Two groups can be distinguished among the hyperosmoregulating, euryhaline crustaceans that can migrate between seawater and dilute ambient media. Some hyperosmoregulators show significant changes of the hemolymph osmolality when they are confronted with increasingly dilute ambient media and usually do not succeed to invade freshwater (example: *Carcinus maenas;* [1660]). Other euryhaline hyperosmoregulators stabilize their hemolymph osmolality in increasingly dilute ambient media and succeed to survive in freshwater (example *Eriocheir sinensis*). Many euryhaline hyperosmoregulators become isosmotic in seawater or hypersaline ambient media. This group has been termed "hyperosmotic-isosmotic" regulators and is distinguished from "hyperosmotic-hypoosmotic" regulators (1087). Species of the latter group of Crustacea behave like hyperosmoregulators in dilute environments, but maintain lower osmotic concentrations in the body fluids when migrating to seawater or hypersaline waters. Probably

the most prominent example of these crustaceans is the brine shrimp, *Artemia salina*, maintaining a remarkably hypoosmotic hemolymph up to external salinities of 30% NaCl (360).

Intracellular Osmoregulation

Changes of hemolymph osmolality can be very large in crustaceans that migrate between ambient media of different osmotic strength. Such changes have an evasive nature. If hyperosmoregulators reduce the osmotic gradient across the body surface, the passive loss of salt and the passive influx of water are reduced, decreasing the burden on the energy-consuming, compensatory mechanisms (active salt absorption and urine production; see below). In this regard it is not surprising that freshwater crustaceans usually show the lowest hemolymph osmolalities. On the other hand, the sometimes dramatic changes of hemolymph osmolalities observed in animals that migrate between ambient media of different osmotic strength pose the question of how the cells respond to this challenge. Two characteristics of animal cell membranes are important in this regard: significant water permeability and the inability to withstand pressure gradients. Consequently, intracellular and extracellular osmolality must be matched to avoid cell damage. The regulation of the intracellular osmotic strength in response to extracellular changes is called intracellular osmoregulation or cell volume regulation. Even in isosmotic extracellular medium, animal cells must actively maintain their volume by using the Na^+/K^+-ATPase to create a situation called double-Donnan or pump and leak (cf. [1124]). Because of the high water permeability of cell membranes, the cell volume rapidly changes when cells are exposed to an anisoosmotic extracellular medium. Two basic strategies are known to maintain the cell volume in such a situation. As a very rapid response to cell volume changes, animal cells transport inorganic ions into or out of the cell, and water follows osmotically, adjusting and maintaining the cell volume. This rapid mechanism of cell volume regulation, termed regulatory volume increase (RVI) or regulatory volume decrease (RVD), depending on a hypoosmotic or a hyperosmotic challenge, was mainly studied in isolated cells of vertebrates (for a recent review, see [783]); similar investigations with crustacean cells have not been conducted. The second basic strategy of intracellular osmoregulation or cell volume regulation is based on a response of the pool of organic osmolytes to a change of the extracellular osmolality. A very informative study of the response of the intracellular amino acid pool was conducted with Chinese crabs (*Eriocheir sinensis*). The results are summarized in Fig. 5 (from [611]).

Chinese crabs adapted to seawater were transferred to freshwater, and the water content and amino acid concentration of muscle cells was monitored. The tissue water content showed a rapid peak before it stabilized after about one day. Concomitantly, the amino acid content of the muscle tissue dropped, indicating that the reduction of intracellular amino acids stabilized the cell volume. At the end of the experiment,

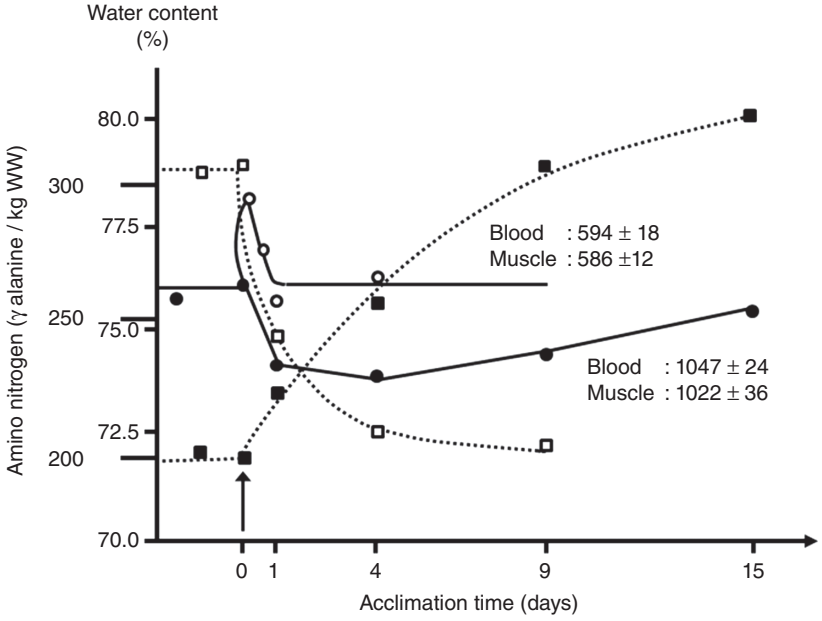

Figure 5 Volume regulation in muscle cells of Chinese crabs (*Eriocheir sinensis*). Animals acclimated to seawater were transferred to freshwater, and the tissue water content (open circles) and the amino acid concentration (open squares) were monitored over time. Hemolymph and cell osmolality amounted to approximately 1,000 mosmol kg^{-1} in seawater and dropped to approximately 590 mosmol kg^{-1} in freshwater. A different group of animals was acclimated to freshwater and then transferred to seawater, and the tissue water content (closed circles) and the amino acid concentration (closed squares) were again monitored. Redrawn after (611), with permission of John Wiley and Sons Ltd.

hemolymph and muscle tissue reached a new osmotic equilibrium at approximately 600 mosmol/kg. When the experiment was reversed, transferring freshwater-adapted crabs to seawater, a transient drop of the tissue water content was observed. During the phase of recovery, amino acids in muscle tissue were increased until a new equilibrium was reached with an osmolality of approximately 1,000 mosmol/kg in hemolymph and muscle tissue. Similar studies confirmed the role of amino acids in intracellular volume regulation in isolated tissue and in other crustacean species. Apart from the amino acids involved (taurine and some quaternary ammonium derivatives are also of some importance), the significance of transport of amino acids into or out of cells, of storage/liberation of amino acids in/from protein, as well as of their synthesis/breakdown has been addressed in (611-613, 1635). Whether cell volume regulation based on the transport of inorganic ions (see RVD and RVI above) is of importance in Crustacea to stabilize the cell volume during the first phase after an osmotic challenge remains to be addressed in the future.

Body Surface Permeabilities

Another evasive strategy of osmotic regulation is the reduction of the permeability of the body surface for passive flows of salt and water. Apart from studying hemolymph osmolalities and ion concentrations, whole animals were also used to determine whole body surface permeability, cf. (941, 1085). Not surprisingly, osmoconforming crustaceans show the highest body surface permeabilities for water and salt. Euryhaline hyperosmoregulators like *Carcinus maenas* have significantly reduced permeabilities of their body surface (1660). Even lower are the permeabilities of animals that succeed in migrating to freshwater (*Eriocheir sinensis*; [1659]), and of animals that continuously inhabit freshwater, such as freshwater crabs (*Potamon niloticus*; [1658]) or crayfish (*Astacus fluviatilis*; [215]). Also hypoosmoregulating crustaceans like *Artemia salina* (1717) or fiddler crabs (65) were found to have significantly reduced water permeabilities of the body surface (cf. [941]). Changes of body surface permeabilities seem to appear over a time-course of days. However, the structural and mechanistic changes involved are poorly understood and require further investigation.

Water balance

Reduced osmotic gradients and body surface permeabilities are evasive strategies of osmotic regulation. The compensatory mechanisms—active salt absorption and urine production in hyperosmoregulators, and active salt secretion and drinking in hypoosmoregulators—have also been studied on whole animals.

The first compensatory mechanism in osmotic regulation discussed here is the adjustment of water volumes. For hyperosmoregulators, a passive gain of water has to be compensated for by excretion of urine. Crustaceans use their antennal glands, also called green or renal glands, or the maxillary glands to produce urine. It should be noted that crustaceans are ammoniotelic. Instead of the antennal and maxillary glands, the gills are the primary site of excretion of nitrogenous waste (cf. the section below on Excretion). Anatomically, the crustacean antennal glands consist of a coelomic end sac, an excretory tubule, and a short duct that opens to the environment either at the base of the second antenna (antennal glands) or at the base of the second maxilla (maxillary glands; cf. [556]). It was observed that glucose appears in crustacean urine after injection of phlorizin (an inhibitor of glucose reabsorption), and that inulin is concentrated (1554, 1555). From these observations it was concluded that filtration is the mechanism producing the primary urine and that the final urine is produced by reabsorption and secretion along the tubular gland. Therefore, the antennal glands of Crustacea are anatomically and physiologically similar to the vertebrate nephron (1549). It has been shown that the urine volume responds significantly in weak hyperosmoregulators to the increased passive water influx in dilute ambient medium. For example, osmoconforming *Carcinus maenas* produced 4.4 ml kg^{-1} h^{-1} when in seawater, but 21 ml kg^{-1} h^{-1} when hyperosmoregulating in 40% seawater (138). Similar results were obtained with other euryhaline crustaceans (e.g., 1088, 1405, 1966). On the other hand, urine flow in freshwater crustaceans can be very small (e.g., 215, 1550, 1658). In osmoconformers and many hyperosmoregulators, the urine is isosmotic with the hemolymph (941, 1151), and weak hyperosmoregulators lose significant amounts of salt with the large volume of urine. On the other hand, strong hyperosmoregulators produce much lower volumes of urine, which is certainly related to the low water permeability of their body surface (see above). Even though freshwater crabs produce isosmotic urine, the salt loss with the urine is reduced because of the low volume of urine production. Some strong hyperosmoregulators, especially crayfish, have evolved the capability of anisoosmotic reabsorption of salt and the production of dilute final urine (1151, 1404, 1551, 1633; see below).

In seawater, the urine volume of crustaceans can be very low and varies between 3% and 15% of the body weight per day (cf. [1085]). For hypoosmoregulating Crustacea, some studies indicated that the urine volume further decreases (669), whereas in others the urine volume was lowest when the animals were isosmotic with the external medium (1405). Hypoosmoregulators should cover their passive water loss by drinking like marine bony fish does (see the section on Agnatha and Pisces below). In some hypoosmoregulating species, drinking was demonstrated as a means of water balance (65, 371, 694, 1717).

Active NaCl transport

With regard to hyperosmoregulating Crustacea, a most remarkable series of studies by Shaw found that the external sodium concentration for half-maximal sodium uptake was different for different crustaceans: *Carcinus maenas* (~20 mM; [1660]), *Eriocheir sinensis* (~1 mM; [1659]),

Gammarus duebeni (1.5 mM; [1661]), *Astacus pallipes* (0.2-0.3 mM; [1657, 1658]), and *Gammarus pulex* (0.1 mM; [1661]). Again, these results allowed to distinguish between different groups of hyperosmoregulators. Weak hyperosmoregulators are those animals that have a higher concentration for half-maximal saturation of salt uptake, higher body surface permeabilities for salt and water, and higher changes of hemolymph osmolality when exposed to different ambient media (see above). Strong hyperosmoregulators have a low concentration for half-maximal saturation of salt uptake, lower body surface permeabilities for salt and water, and lower changes of hemolymph osmolality when exposed to different ambient media (see above). Studies with whole animals also generated some insights into the mechanisms of active NaCl absorption in hyperosmoregulating crustaceans. Krogh (973) showed that the Chinese crab (*Eriocheir sinensis*) can absorb sodium and chloride independent from each other. In later studies that used inhibitors of transporters developed in the meantime (479, 931, 942, 2068, 2072), further insights were obtained with regard to the mechanisms of active NaCl absorption in strong hyperosmoregulators (see below).

In another series of remarkable studies, Croghan (357-361) generated data for the active NaCl secretion in hypoosmoregulating *Artemia salina* that are compatible with the mechanisms of active NaCl secretion as they have been described for seawater fish gills, elasmobranch rectal glands, and the salt glands of birds and reptiles, as discussed in the respective sections below. These studies also showed evidence for the involvement of the gills and the gut in active salt secretion. Unfortunately, much more conclusive evidence for active salt secretion in other crustacean species could never be collected (see also below).

Ion and acid/base regulation

Numerous studies with whole animals focused on ion regulation in Crustacea. Calcium regulation is of particular importance for the maintenance of the exoskeleton. During intermolt, small calcium losses with the urine are probably compensated for by the calcium content of the food and/active calcium absorption by the gills. During premolt, calcium is absorbed from the exoskeleton and stored in gastroliths. Some calcium is also excreted by the gills and by the hepatopancreas. During postmolt, calcium is secreted and inserted into the new exoskeleton. During this phase, the calcium from the stores is recovered and additional calcium is supplied by calcium absorption from the food in the hepatopancreas and by absorption from the ambient medium by the gills. This scenario is based on studies with whole animals and with biochemical and molecular approaches using tissue from the antennal glands, the hepatopancreas, and the gills (for reviews, see 12, 1304, 1969, 1970, 1977).

Numerous studies focused on acid/base regulation in Crustacea (for reviews, see 241, 744, 1868, 1869, 1974). Acid/base regulation is linked to NaCl regulation. On one hand, it has been clearly shown that changes of ambient ion concentrations and salinity affect acid/base regulation (735, 1867, 1869, 1971, 1981) and vice versa (1864). On the other hand, it was shown that exchange of acid for sodium and base for chloride can operate independent of each other in crayfish (479).

Antennal Glands

Although transport was never studied with isolated and perfused antennal glands, measurements of osmotic and solute concentrations along excised antennal glands allowed some more detailed insights. As mentioned above, most Crustacea produce isosmotic urine. However, some freshwater crustaceans evolved the capability to reduce their salt loss by producing dilute urine. In the crayfishes *Austropotamobius pallipes pallipes* and *Orconectes virilis*, the most dilute urine is found in the bladder (1547, 1551, 1552). Anisoosmotic salt absorption seems to occur in a part of the distal tubule, which is especially long in crayfish (1436). For some crustaceans, ratios of urine and hemolymph osmolality above 1 were reported (e.g., 641). However, the only hypoosmoregulator for which significantly concentrated urine was reported is the brine shrimp *Artemia salina* (cf. 1404).

Active NaCl transport in antennal glands

Apart from their evident function in the regulation of hemolymph volume (see above), antennal glands of Crustacea are very significant in ion regulation. The primary urine can be considerably modified by glucose and amino acid reabsorption (84, 85, 1063, 1554), secretion of magnesium and sulphate (262, 555, 787, 920, 933, 1063, 1089, 1256, 1404, 1602, 1625, 1633, 2066), absorption of potassium (1063, 1404, 1552) and reabsorption or secretion of calcium (9, 1063, 1404, 1568, 1972, 1975, 1976, 2067). The inulin ratio between urine and hemolymph is usually above 1, indicating water reabsorption following active absorption of solute, and reflects a mechanism to reduce urine volume (1556).

The Na^+/K^+-ATPase seems to be the major ion pump energizing transport in antennal glands. The activity of the pump has been measured and its basolateral localization has been verified (e.g., 921, 1436, 1607). Apical membrane of labyrinth and bladder were used to prepare membrane vesicles and characteristics, and kinetics of specific transporters were determined (10, 84, 85). Molecular biology techniques allowed gene sequencing and the study of the regulation of transporters in the antennal glands (1973).

The Gut and Hepatopancreas

The gut and hepatopancreas of crustaceans reflects a considerable surface in contact with the environment. However, a considerable contribution of the gut and hepatopancreas in osmoregulatory processes has never been shown (1151). The hepatopancreas is certainly involved in the absorption of nutrients (11, 1910), calcium regulation during the molt

cycle (12) and the sequestration and detoxification of heavy metals (13).

Crustacean Gills

The most important organs of osmotic regulation in Crustacea are the gills. First clear evidence for the dominant role of the gills in active NaCl absorption was presented by Koch et al. (953), measuring sodium absorption with isolated gills. In the meantime it has been clearly established that the gills are major organs of osmotic and ionic regulation in Crustacea (for some reviews, see 556, 740, 934, 1151, 1418, 1421, 1476). In all cases, the gills represent a significantly increased surface area in contact with the ambient medium and protected inside a gill chamber. Crustacean gills are multifunctional organs involved in osmotic and ionic regulation (for references, see above), gas exchange (1211), Ca^{2+} regulation (12, 1304, 1969, 1977), pH regulation (241, 744, 1868, 1869, 1974) and excretion of nitrogeneous waste (584, 1601, 1950). The number of paired gills, their location of attachment (pleurobranchs, arthrobranchs and podobranchs), as well as the extent and anatomical form of surface extension (phyllobranchiate, trichobranchiate and dendrobranchiate gills) varies between groups and has been extensively described in Taylor and Taylor (1811).

After the progress by Koch et al. (953), the technique of measuring ion fluxes and transepithelial voltages with isolated and perfused gills was further developed, and active NaCl absorption was demonstrated for a number of species (e.g., 130, 925, 1114, 1150, 1455, 1456). For crabs, it became evident that only the posterior gills were involved in active NaCl absorption (1419, 1420). In many studies the transepithelial voltage was measured with isolated gills. However, often different solutions were used to perfuse and bath the gills, often making it impossible to analyze whether the measured voltages reflected active transport or diffusion potentials caused by the concentration gradients and ion-selective paracellular pathways. Only later the electrogenic nature of active NaCl absorption across crustacean gills was clearly shown (451, 614, 1114, 1455, 1456, 1689). For active NaCl secretion in hypoosmoregulating Crustacea, the use of isolated and perfused gills did not yet bring much further insights (1114). Isolated and perfused crustacean gills proved very useful in many cases, but in order to unambiguously analyze the mechanisms involved in transbranchial NaCl transport, the technique needed to be refined. In 1989, a technique was introduced that made use of the lamellar structure of crab gills (1640). Single lamellae were isolated and split and the resulting small sheet of epithelium covered on the apical side with cuticle could be mounted in small Ussing chambers. With split gill lamellae, further methodological advancements like voltage clamp, the use of microelectrodes (1374) and current noise analyses (2070) became possible and were used. The electrical characteristics of the gill epithelium could be analyzed and the mechanisms of active, transbranchial NaCl absorption could be uncovered. Later, the same technique was used for the pleopods of isopods (1471) and for epipodites of lobsters (1107, 1109).

Active transbranchial NaCl absorption in strong hyperosmoregulators

Consistent with the observations of Krogh (973) on whole animals, active sodium and chloride absorption across the gills of Chinese crabs (*Eriocheir sinensis*) can be measured independent of each other (see Fig. 6).

When mounted in an Ussing chamber, the gill epithelium of the Chinese crab generated a positive short-circuit current (I_{SC}; reference electrode in the internal bath. A positive Isc reflects absorption of cations or secretion of anions.) in the absence of chloride in the external bath. This I_{SC} was sodium dependent and could be inhibited with internal ouabain, a blocker of Na^+/K^+-ATPase, or with submicromolar concentrations of amiloride in the external bath. Amiloride, a blocker of different sodium transporters, induced current-noise. Analyzing this amiloride-induced current-noise revealed the presence of epithelial sodium channels (2070). Single-channel currents and the density of channels in the apical membrane were determined. In the absence of sodium in the external bath, the gill epithelium of Chinese crabs generated a negative I_{SC} (1374, 1379). This current was chloride dependent and could be inhibited with external DIDS/SITS or bafilomycin, or by using chloride channel blockers in the internal medium. Inhibition of carbonic anhydrase abolished the negative I_{SC}, whereas ouabain did not affect it. Together, the results indicated a mode of NaCl absorption very similar to the mechanisms described for freshwater amphibia and fish (see Fig. 7), involving apical sodium channels, chloride/bicarbonate exchangers and V-type proton pumps, basolateral Na^+/K^+-ATPase, potassium and chloride channels, and intracellular carbonic anhydrase that rapidly supplies protons and bicarbonate ions for the apical transporters.

With a paracellular leak conductance below 1 mS cm^{-2}, the gill epithelium of Chinese crabs is clearly a tight epithelium. Using whole animals (479, 931, 938, 942, 2068, 2072) or split gill lamellae (1375, 1949), results were obtained, indicating that the same or a very similar mechanism of active NaCl absorption is used by strong hyperosmoregulating Crustacea in general.

Active transbranchial NaCl absorption in weak hyperosmoregulators

The first studies of split gill lamellae of a weak hyperosmoregulator were conducted with *Carcinus maenas* and showed significant differences (1383, 1557) to strong hyperosmoregulators. Split gill lamellae generated a negative I_{SC} that depended on the presence of both sodium and chloride in the external bath (see Fig. 8).

The current was inhibited with internal ouabain, barium/caesium or chloride channel blockers, and with external caesium. This suggested a mechanism of NaCl absorption

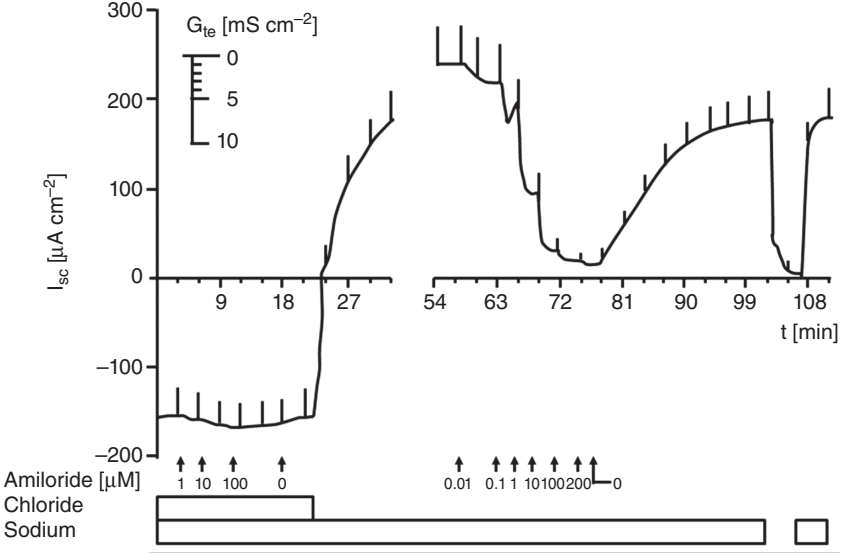

Figure 6 Independent absorption of Na^+ and Cl^- in the low-conductance epithelium of posterior gills of the strong hyperosmoregulator *Eriocheir sinensis*. The figure shows a representative time course of the short-circuit current (I_{SC}), demonstrating the negative I_{SC} (reflecting active, Na^+-independent Cl^- absorption, further characterized in [1374]) and the positive I_{SC} (reflecting active, Cl^- -independent Na^+-absorption, further characterized in [2070]) across a split gill lamella. The I_{SC} deflections are due to 10 mV voltage pulses and are directly proportional to the transepithelial conductance (G_{te}; see scale on the upper left, redrawn after [2070]). In the presence of high external Cl^-, most apical Na^+ channels are closed, and even high concentrations of external Amiloride hardly affect current and conductance. At t = 23 min: In the absence of external Cl^- (substitution with gluconate, or at low external Cl^-; cf. [1372]), apical Na^+ channels open and external amiloride reduces current and conductance in a concentration-dependent way. At the end of the experiment, the Na^+-dependence of current and conductance is shown by additional substitution of Na^+ (TRIS). The low conductance at maximal external amiloride is a measure for the paracellular conductance.

similar to the mode of NaCl absorption in the thick ascending limb of Henle's loop in the mammalian nephron where a Na^+-$2Cl^-$-K^+ cotransporter plays a central role (Fig. 9). Inhibitors of the cotransporter had no effect on split gill lamellae, but the ratio of net influx of chloride to current (approximately 2:1) and the ratio between net influxes of sodium and chloride (approximately 1:2) were taken as considerable evidence for the presence of such a cotransporter, which was later found with molecular techniques (1848).

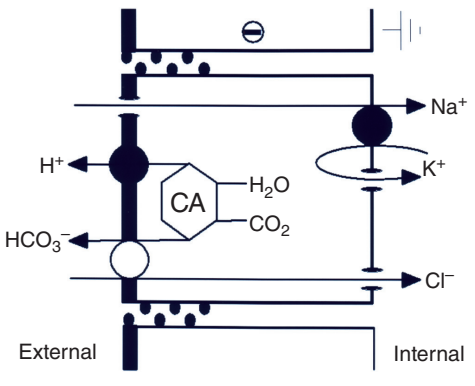

Figure 7 Mechanism of transbranchial NaCl absorption in strong hyperosmoregulors, from (556). For further details, see the text.

Not only the mechanism of active NaCl absorption but also the basic epithelial characteristics were significantly different when compared to strong hyperosmoregulators. Split gill lamellae of weak hyperosmoregulators have a high conductance (40-60 mS cm^{-2}), which is dominated by the conductance of the paracellular pathway (1383, 1384, 1557). Using different experimental approaches, this mechanism was confirmed for other weak hyperosmoregulators (1384, 1455, 1456), including isopods (1471) and lobsters (1107, 1109). However, it seems that different species also apply electroneutral NaCl absorption via apical sodium/proton and chloride/bicarbonate exchangers to varying degrees (cf. 556).

Active transbranchial NaCl absorption in land crabs

In land crabs, the gills play a somewhat unexpected role in osmotic regulation. These animals live in humid areas and their major osmotic problem is therefore not water loss. Instead, the loss of salt with the isosmotic urine is more critical, especially because land crabs have high hemolymph salt concentrations. To reduce the loss of salt, the urine is either ingested or passed over the gills where NaCl is reabsorbed (1254, 2019, 2020).

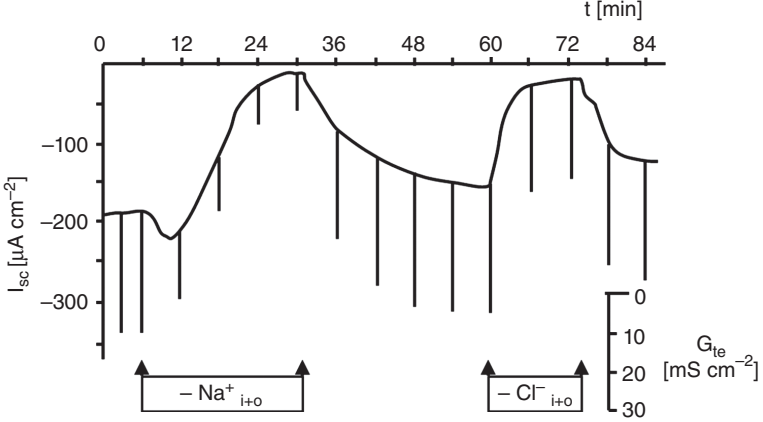

Figure 8 Coupled absorption of NaCl in the high-conductance epithelium of posterior gills of the weak hyperosmoregulators *Carcinus maenas*. The figure shows a representative time course of the short-circuit current (I_{SC}), demonstrating the effects of either Na^+ substitution (choline) or Cl^- substitution (gluconate) on both sides of a split gill lamella mounted in a modified Ussing chamber. Current deflections are due to 5 mV voltage pulses and are directly proportional to the transepithelial conductance (G_{te}; see scale for G_{te} in lower right, redrawn after [1383]). The larger G_{te} decrease in the absence of Na^+ indicates that the paracellular conductance is selective for Na^+.

Active transbranchial NaCl secretion

In hypoosmoregulating Crustacea, salt must be actively secreted. Results obtained with hyporegulating fiddler crabs indicated that active NaCl secretion takes an extra-renal pathway (65, 510, 641). In *Artemia salina*, active NaCl secretion was related to the gills (358), and ultrastructural analyses supported the involvement of the gills for other crustaceans as well (1113, 1170). Isolated gills of hyporegulating *Neohelice* (*Chasmagnathus*) granulata generated a large net efflux of sodium when the gills were perfused and bathed with identical salines (1114), and this active secretion depended on a functioning Na^+/K^+-ATPase. However, the complete mechanism of active, transbranchial NaCl secretion in hyporegulating crabs is unknown. Interestingly, the transepithelial voltage was outside positive, which is opposite to the expectation for a mechanism similar or identical to hyporegulating marine fish. Future studies must show whether Crustacea evolved mechanisms of active NaCl secretion different from those of the vertebrates.

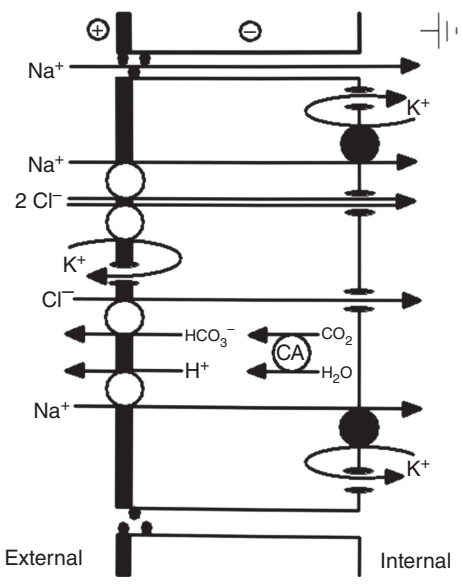

Figure 9 Mechanism of transbranchial NaCl absorption in weak hyperosmoregulators (from [556]). For further details see the text.

Many transporters involved in transbranchial ion transport, including ion pumps, cotransporters, exchangers, ion channels and the carbonic anhydrase, have been identified, characterized and localized with various techniques, including pharmacology, biochemistry, histochemistry, immunohistochemistry and molecular biology (cf. 556, 1110, 1151, 1418, 1421, 1846, 1848). The influence of the gill cuticle on transbranchial ion transport (reviewed in [1050]) relates to an additional permeability barrier and the generation of an additional compartment, the subcuticular space. Although the electrical resistance of gill cuticles of actively transporting gills is low when compared to the resistance of the epithelium, ion selectivity and rectification that were observed in a number of cases (56, 1051) could interfere with the overall transport process. More detailed studies are required in the future to elucidate the role of the gill cuticle. In any case, it is worthwhile to underline that certain drugs have been shown to interfere with the cuticle (1380), which may result in misinterpretations in pharmacological studies.

Some anatomical and functional specializations in the gills of crabs and crayfish have been discussed in a recent review (556).

Modulation of Osmotic and Ionic Regulation by Hormonal and Non-Hormonal Factors Apart from studying the changing capabilities and mechanisms of osmotic regulation during the ontogenetic development of crustaceans (for reviews, see 46, 272, 273), almost all studies of the hormonal and non-hormonal osmotic regulation in crustaceans have been performed with adult animals of euryhaline species. These animals migrate between different salinities and have the capability to acclimate to changes in ambient osmolalities. In some species, such migrations between waters of different salinities are essential prerequisites for a successful larval development. Many studies monitored changes of hemolymph osmolality and ion composition in response to changing ambient osmolalities (cf. 1151; see also above and Fig. 4). Most studies focused on the most prominent organ of osmotic regulation in Crustacea: the gills.

No signs of active NaCl absorption could be detected in osmoconform crustaceans from seawater. In contrast, active NaCl absorption was measured as net influxes of sodium and/or chloride, transepithelial electrical voltages or currents after the animals were acclimated to dilute ambient media (e.g., 1108, 1372, 1373, 1418, 1421). In the Chinese crab *Eriocheir sinensis*, the paracellular conductance was observed to be significantly decreased after acclimatization to dilute ambient media (1372).

Comparing osmoconforming and hyperosmoregulating animals (hyperosmotic-isosmotic regulators) of the same species showed sometimes dramatic changes in gill structure (e.g., 331, 339, 547, 1170, 1214, 1215, 1421). Whereas the gill epithelial cells are thin in osmoconforming animals, the gill epithelium of hyperosmoregulating crustaceans is thick and the cells display extensive systems of apical and basal infoldings. The number and density of mitochondria is usually increased in euryhaline hyperosmoregulators, reflecting a higher energy requirement in these animals. In palaemonid shrimps, significant structural changes can be observed in the epithelial pillar cells and the adjacent septal cells. In some hyperosmotic-hypoosmotic regulators, the structural features typical for hyperosmoregulation do not disappear when the animals are acclimated to seawater or hypersaline ambient media where these animals behave as hyporegulators (533, 1113, 1114, 1170).

Concomitantly with the structural changes, increases in Na^+/K^+-ATPase activity (e.g., 1152, 1153, 1422, 1687, 1741, 1931; reviewed in 1110, 1151, 1418, 1421, 1845) and mRNA expression (845, 1101, 1110, 1115, 1848) have been observed in a number of hyperosmoregulating species. Similarly, augmented carbonic anhydrase activity and mRNA expression has been found (e.g., 156, 157, 731-739, 741-743, 1370, 1460, 1649, 1650). mRNA expression of V-ATPases was increased in the strong hyperosmoregulator *Eriocheir sinensis* (1955). Interestingly, in the hyper-hyporegulator *Neohelice* (*Chasmagnathus) granulata*, mRNA expression of Na^+/K^+-ATPase and Na^+-$2Cl^-$-K^+ cotransporters was dramatically increased in the posterior gills of hyper- and hyporegulating crabs when compared with the gills of isosmotic crabs (1115). This finding indicates the involvement of these transporters in active NaCl absorption when the animals are exposed to a dilute environment and in active NaCl secretion when the animals are hyporegulating in hypersaline ambient medium. In crabs, the structural and functional changes reviewed above are limited to the posterior gill pairs. The anterior gills of crabs do not respond to salinity changes and have apparently no role in osmotic regulation.

In strong hyperosmoregulating Chinese crabs acclimated to freshwater, changes of the hemolymph-side osmolality resulted in alterations of the rates of active absorption of sodium and chloride measured as short-circuit currents (1371). Because increasing the osmolality resulted in a decrease of NaCl absorption and decreasing the osmolality increased active transport, the observation was termed autoregulation. It was assumed that the transport modulation is based on cell volume changes, because the addition of membrane-impermeant sucrose reduced transport rates, whereas the addition of membrane-permeant urea increased them. Another observation with importance for regulatory mechanisms was an interaction between transcellular chloride and sodium absorption (1372). In the presence of low external NaCl, sodium and chloride absorption short-circuit each other. At higher external NaCl concentrations, transcellular chloride absorption can be monitored, whereas transcellular sodium absorption is abolished or significantly reduced. Transcellular sodium absorption becomes visible only when chloride absorption is inhibited. It appears that higher rates of chloride absorption induce an intracellular change of so far unknown nature that inhibits transcellular sodium transport. *In vivo*, NaCl absorption could be rapidly adjusted with the underlying mechanism by modulating the apical sodium permeability and by directing sodium fluxes across the apical membrane (high transport rates in very dilute ambient media) or across the low conductance paracellular junctions (low transport rates at increased ambient NaCl). After acclimation of the animals to brackish waters (9% or 18% salinity) the paracellular conductance was still below 1 mS/cm^2, whereas it significantly increased after acclimation to full-strength seawater. Autoregulation by the hemolymph-side osmolality was also observed in the weak hyperosmoregulator *Neohelice* (*Chasmagnathus*) *granulata* (1860). The results of the latter study indicated that the stimulating effect of reduced hemolymph-side osmolality was based on a Na^+/K^+-ATPase activation and mediated in part by an increased intracellular cAMP concentration. Activation of adenylate cyclase by mechanical stretch of the membrane is indeed known from vertebrates (1935).

Increased levels of cAMP were also found to stimulate transport rates in the strong hyperosmoregulators *Eriocheir sinensis* (1558). Sodium absorption was shown to be stimulated by increasing the number of open sodium channels in the apical membrane, whereas the cAMP-induced stimulation of chloride absorption was accompanied by a significant increase of the electromotive force, suggesting a cAMP-induced increase in V-ATPase activity. Subsequently,

an extract of the eyestalks was shown to mimic these effects (1382), and it was proposed that one of the peptide hormones from the sinus gland may be involved in the regulation of active, transbranchial NaCl absorption in Chinese crabs. Dopamine also induced an increase in intracellular cAMP in the gills of Chinese crabs, stimulating sodium influxes by increasing Na^+/K^+-ATPase activity (1241, 1242). Also, in weak hyperosmoregulators, dopamine was observed to increase intracellular cAMP in the gills and to stimulate active salt absorption via activation of Na^+/K^+-pumps (594, 689, 880, 881, 1730). In the weak hyperosmoregulator *Pachygrapsus marmoratus*, crustacean hyperglycemic hormone (CHH) was shown to stimulate salt absorption (1739), and in freshwater crayfish, CHH injection prevented salt loss after eyestalk removal (1648).

Despite the wealth of data about osmotic and ionic regulation in Crustacea, further, more detailed analyses are necessary for a more complete understanding of the mechanisms of osmotic and ionic regulation and their modulation by hormonal and non-hormonal factors.

Insecta

Design of Insect Osmoregulatory and Excretory systems

The excretory system of insects is comprised of the Malpighian tubules and the gut, especially the hindgut (Fig. 10). Each Malpighian tubule consists of a single layer of squamous epithelial cells that form a blind-ended tube. Tubules range from 2 mm to 70 mm in length, 2 to 250 in number and up to 100 μm in diameter (1438).

Insects provide a study in contrasts with the vertebrates in terms of the sites and mechanisms of urine formation. In most vertebrates, blood pressure is the driving force for ultrafiltration across the glomerulus of the kidney. The resulting primary urine is modified by reabsorption of salts and water in the loop of Henle and the collecting ducts. The exoskeleton precludes the development of significant hemolymph pressure in most insects, so ultrafiltration is not feasible. Instead, formation of the primary urine involves active transepithelial secretion of ions (primarily K^+, Na^+ and Cl^-) into the lumen of the Malpighian tubule and passive flow of osmotically obliged water. The primary urine is modified subsequently by reabsorptive or secretory processes in the hindgut, although in a few species such modification occurs in a downstream segment of the tubule itself. The higher ratio of surface area to volume for tubule cells to tubule lumen, relative to hindgut cells to hindgut lumen, presumably allows for more rapid processing of the urine in insects subject to fluid loading, such as blood-feeding hemipterans and the fruit fly *Drosophila melanogaster*.

The bulk of water, ion and metabolite reabsorption occurs in the rectum, resulting in strongly hyperosmotic or hypoosmotic excreta. In many terrestrial species of insect, the hindgut can recover virtually all the water from the gut contents and fluid secreted into the gut by the Malpighian tubules. The resulting fecal pellets are powder-dry in species such as the mealworm *Tenebrio molitor* (1503) and the firebrat *Thermobia domestica* (1324). High concentrations of solutes, wastes

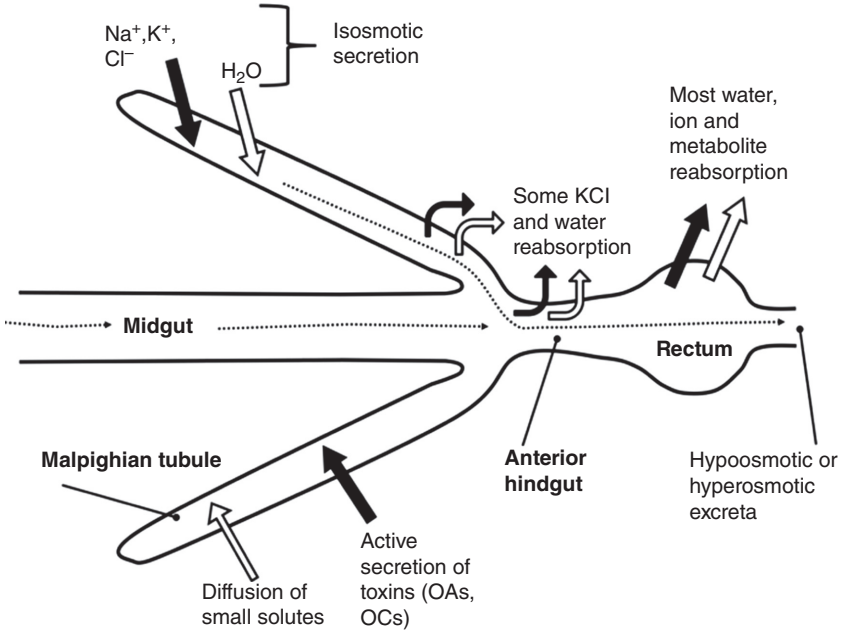

Figure 10 Schematic diagram of the insect excretory system. Active transport is indicated by solid arrows and passive transport is indicated by open arrows. The flows of the gut contents and the fluid secreted by the Malpighian tubules are indicated by dashed arrows. Adapted from Phillips (1438), with modifications. Abbreviations: organic anions: OAs; organic cations: OCs.

and toxins are produced in the lumen as a consequence of water recovery (1443). The cuticular lining of the hindgut thus plays an important role in protecting the transporting cells of the rectal epithelium from lumen contents.

Processes such as desiccation, feeding or osmotic influx across the external surfaces of aquatic species create the need for adjustments of hemolymph fluid and solute levels. However, several features of insect physiology and morphology reduce the evolutionary pressure for rapid alterations of hemolymph composition by the excretory system. First, insects do not depend on a blood-borne respiratory pigment whose effectiveness would be altered by changes in the volume and composition of the extracellular fluids. Instead, respiratory gas exchange is accomplished by the ramifying tubes of the tracheal system whose termini extend into cells and tissues. Second, an effective blood-brain barrier protects the eyes and central nervous system from extracellular toxins or changes in hemolymph ionic composition. Epithelia such as the perineurium, which envelopes the insect central nervous system, are characterized by the presence of effective cell-cell junctions and by inorganic ion and xenobiotic transporters that protect the neuronal tissues (1188, 1857). Third, the waxy cuticle limits water loss, so that terrestrial insects are protected from rapid desiccation in spite of their large surface area-to-volume ratio.

Given the coevolution of insects and flowering plants and the large number of plant-derived toxins that have evolved to minimize herbivory, a key role of the insect excretory system is to automatically eliminate novel toxins. The Malpighian tubule provides a means for removal from the hemolymph of all soluble substances of low molecular weight, and in this respect it provides analogies with both the glomerulus and the tubule of the vertebrate nephron. It is somewhat unexpected, therefore, that the absolute permeability of the Malpighian tubule epithelium of insects is quite low when compared to the excretory epithelia of other invertebrates and the vertebrate kidney. Given the need to clear toxic compounds from the hemolymph, this characteristic seems paradoxical (1346, 1706). However, it is the area for passive permeation, namely the paracellular clefts, that is restricted, rather than the permeability of these sites. Adjacent cells in insect epithelia, such as the Malpighian tubule, are separated by septate junctions rather than the occluding, or tight, junctions of transporting epithelia in vertebrates (988). Although the ratio of the areas of the basal side of the cells (i.e., the side facing the hemolymph) and the intercellular cleft is 3,000:1, the ratio of transcellular to paracellular area increases to 120,000:1 because of the 40-fold amplification of basal surface area created by membrane folding and convolution (1706).

The rates of passive diffusion of solutes across the tubule epithelium vary inversely with molecular mass (372, 1135). Uncharged solutes that have a molecular mass greater than that of sucrose, as well as charged solutes, are excluded from the tubule cells and can cross only via the intercellular junctions. Uncharged solutes whose molecular mass is less than that of sucrose, including relatively large molecules such as mannitol, cross into the lumen of the upper Malpighian tubule of *Rhodnius* by a cellular route (1346). Rapid permeation by the transcellular route in *Rhodnius* is a consequence of the large area of cell membrane available.

Whereas the vertebrate glomerulus passes compounds as large as inulin, whose molecular weight is $\sim$ 5,000, the Malpighian tubules of insects secrete fluid that contains even such small molecules as sugars and amino acids at concentrations much below those in the hemolymph. Toxic molecules, even those of moderate size, can diffuse through the intracellular clefts and are removed passively, albeit slowly, from the insect's hemolymph (1127). One advantage of this arrangement is that less energy is required to produce the primary excretory fluid and to reabsorb useful substances from it. Also, high concentrations of molecules such as amino acids, trehalose and lipids can be maintained in circulation. Trehalose concentrations may exceed 100 mM in the hemolymph of flying insects, where the sugar is used as fuel for the flight muscles (1962). Many species of butterflies, ants and beetles contain levels of amino acids in the hemolymph as high as 100-200 mM (1128). In addition, hormones that are released into circulation will also be removed slowly by the excretory system, and the amounts that must be released may therefore be reduced.

Insects can eliminate excess fluid at very high rates during diuresis, yet they lose only trace amounts of hemolymph solutes. Given that many hemolymph solutes of even quite small molecular size only appear in the filtered fluid at concentrations in the 10%-50% range of those in the insect's hemolymph, reabsorption of such solutes need only occur in insects at about 1%-2% of the rate required in many vertebrates, which filter their extracellular fluid 10-20 times more rapidly than do most insects. There is thus a large saving in the energetic costs associated with reabsorption of solutes from tubule lumen (1127).

In addition to passive diffusion of solutes from hemolymph to tubule lumen, active mechanisms rapidly transport a wide range of solutes. The tubules reabsorb useful compounds, such as glucose (951), and actively secrete organic cations, organic anions, urate, phosphate, magnesium, sulphate, sulphonates, acylamides, alkaloids and glycosides (1075, 1134, 1334, 1536, 1588, 1844). Studies using tubule-specific DNA microarrays reveal that the Malpighian tubule is an epithelium that is richly endowed with solute transporters, including transporters for many potential toxins (447). The comprehensive and paradigm-shifting research of Dow, Davies and coworkers (285, 446, 447) has documented the enrichment of solute transporters in the tubule genome and suggests a reinterpretation of the primary physiological role of the Malpighian tubules. Rather than just regulating inorganic ion levels in the hemolymph and whole animal water balance—tasks that may be done downstream in the hindgut—the primary role of the tubules may be the regulation of organic solute levels in the hemolymph. Indeed, Dow and Davies (447) point out that in view of electrophysiological data indicating that the tubule is analagous to a vertebrate

tight epithelium (125), its apparent leakiness may stem from high levels of transcellular organic solute transport, rather than paracellular diffusion.

Lastly, the insect excretory system shows complex interactions between the rates of inorganic ion transport, fluid secretion and the elimination of toxins. In particular, a high degree of phenotypic plasticity is seen when insects are exposed to dietary toxins. When *Drosophila*, for example, are reared on diets enriched in organic anions such as salicylate or fluorescein for one day, there is an increase in fluid secretion rate of up to threefold and an increase in organic anion transport of up to fivefold. Since fluid secretion is a passive osmotic consequence of the transport of K^+, Na^+ and Cl^-, these findings indicate that dietary toxin exposure leads to an upregulation of the expression and/or activity of transporters for both organic anions and for inorganic ions (265, 1590). Similarly, when insects are exposed to dietary or environmental salt stress, there is an increase in the capacity to secrete the ion present in excess (443, 1287). For *Drosophila*, the increase in K^+ secretion in response to K^+-rich diets is also associated with a 60% increase in the fluid secretion rate (1288). Earlier studies have shown that the capacity to eliminate uric acid, organic anions or Ca^{2+} increases in tubules isolated from the blood-feeder *Rhodnius* at intervals after the blood meal corresponds to the appearance of these ions in the hemolymph (1130, 1139, 1334). It thus appears to be a general finding that insect Malpighian tubules show pronounced phenotypic plasticity in response to dietary loading with toxins or ions. The epithelium is effectively remodeled so that its capacity to secrete compounds present in excess is increased. Presumably this remodeling is advantageous in that there is not the high metabolic cost of maintenance of transporters when they are not required.

Secretion of Physiological Ions by Insect Malpighian Tubules

Malpighian tubules composed of a single cell type

Much of our knowledge of tubules composed of a single cell type comes from studies of the blood-feeding hemipteran *Rhodnius prolixus*, the ant *Formica polyctena* and the omnivorous orthopteran *Acheta domesticus*. When stimulated with diuretic factors, the Malpighian tubules of *Rhodnius* secrete fluid at prodigious rates, equivalent to each cell secreting a volume of fluid equal to its own volume every 15 seconds and an amount of Cl^- equivalent to the cellular Cl^- content every 3 seconds (1140, 1340). Together, the four Malpighian tubules eliminate urine at a rate equivalent to the insect's unfed body weight every 20 to 30 minutes. The large diameter (90 μm) and length (30 mm) of the secretory segment facilitates setting the tubules up in the *in vitro* Ramsay assay (Fig. 11), where they secrete fluid at ~70% of the *in vivo* rate. The large cells (~ 100 μm) in *Rhodnius* tubules facilitate impalement with double-barrelled ion-selective microelectrodes. Such measurements allow the electrochemical

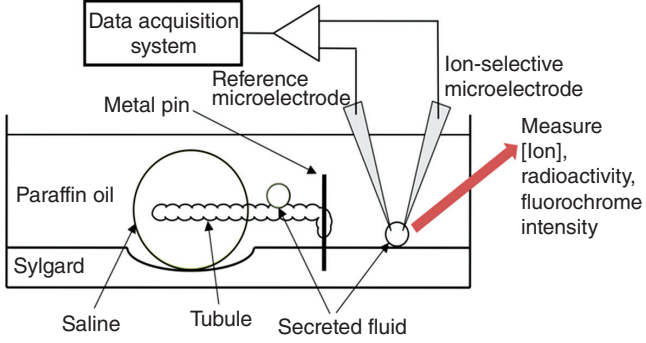

Figure 11 Schematic of the Ramsay assay for measurement of Malpighian tubule fluid secretion rate and secreted fluid solute concentration. An isolated Malpighian tubule is placed in a drop of saline under paraffin oil, and the open end of the tubule is secured to a pin embedded in the Sylgard-lined bottom of the petri dish. Secreted fluid collects at a puncture in the wall of the tubule between the bathing saline and the pin. Secreted fluid ion concentration ([Ion]) is measured using ion-selective microelectrodes (1288), atomic absorption spectroscopy (923) or energy-dispersive X-ray microanalysis (1902, 1998). Organic solute concentrations can be determined using liquid scintillation spectrometry of radiolabeled solutes (1536) or by confocal laser-scanning microscopic measurement of fluorescence intensity of droplets collected in optically flat glass capillaries (1023). Solute secretion rate (flux) is typically in the femtomol min^{-1} to picomol min^{-1} range and is calculated as the product of fluid secretion rate (nl min^{-1}) and solute concentration (μmol l^{-1} to mmol l^{-1}).

gradients for Na^+, K^+, Cl^- and H^+ across the basolateral and apical membranes to be measured. In conjunction with measurements of ion activities in the tubule lumen, these data are essential for evaluating the thermodynamic feasibility of specific channels and other membrane transporters such as Na^+/H^+ exchangers and Na^+-$2Cl^-$-K^+ cotransporters (821, 822, 824).

Rhodnius feeds on blood that is approximately 14% hypoosmotic to its hemolymph osmolality of 370 mosmol kg^{-1} (1133). Homeostasis is maintained by producing hypoosmotic urine through a two-stage process: Na^+, K^+, Cl^- and water are secreted across the upper Malpighian tubule and K^+ and Cl^- but not water are reabsorbed downstream in the lower Malpighian tubule, as described in a subsequent section.

The upper segment of the Malpighian tubules of *Rhodnius prolixus* secretes fluid containing approximately 100 mM NaCl and 80 mM KCl during diuresis (1133). Secretion of ions and osmotically obliged water by tubules of *Rhodnius* and other species is driven primarily by an apical vacuolar-type H^+-ATPase (1137, 1992). Aquaporins are present in both the upper secretory tubule and the lower Malpighian tubule (471), and expression increases in response to blood feeding or treatment of isolated tubules with serotonin or cAMP (1178).

Current models propose that all ion transport is transcellular (578, 821, 824). Paracellular transport of Cl^-, as proposed for mosquito tubules composed of two cell types (see below), is precluded by the lumen-negative potential in *Rhodnius*. It is suggested that electrogenic transport of H^+ from the cell to the lumen energizes amiloride-sensitive exchange of

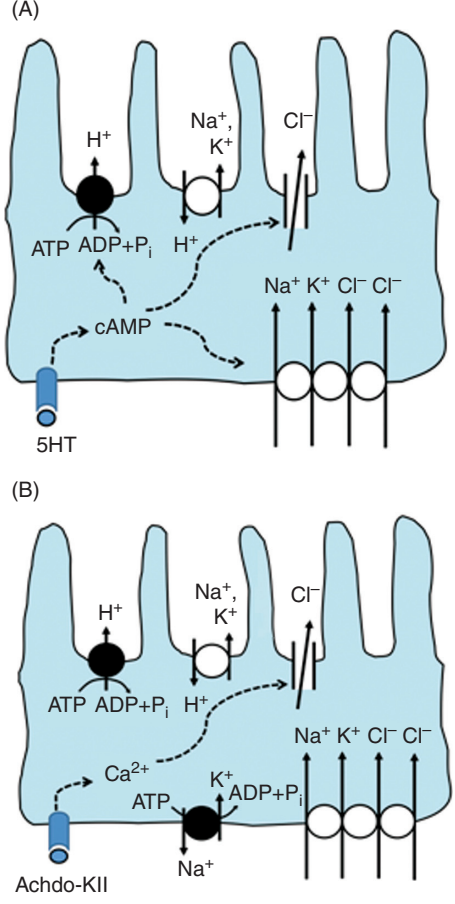

Figure 12 Schematic of ion transport processes involved in fluid secretion by (A) 5HT-stimulated upper tubules of *Rhodnius* (822, 1342) and (B) Achdo-KII-stimulated principal cells in the main segment of cricket Malpighian tubules (316). Dotted lines are used to illustrate the intracellular signaling pathway. Solid circles represent primary active transport processes, whereas open circles are used to represent cotransporters and antiporters. The dark blue symbols represent the receptors for 5HT and for Achdo-KII and their associated ligands.

cytoplasmic K^+ and/or Na^+ for luminal H^+. The primary route of entry of Na^+, K^+ and Cl^- is through a bumetanide-sensitive basolateral Na^+-$2Cl^-$-K^+ cotransporter (Fig. 12A).

A characteristic triphasic change in transepithelial electrical potential (TEP) has been reported when fluid secretion by isolated Malpighian tubules of *Rhodnius prolixus* is stimulated by serotonin (1345). From the initially negative value in unstimulated tubules (−25 mV, lumen-negative), TEP shifts to −33 mV in phase 1, −30 mV in phase 2 and −32 mV in phase 3. The results of ion substitution and addition of specific pharmacological reagents suggest that the three phases of the response of TEP to serotonin correspond to sequential activation of an apical Cl^- channel, an apical V-type H^+-ATPase and a basolateral Na^+-$2Cl^-$-K^+ cotransporter. Bathing tubules in Cl^--free saline before and during 5-HT stimulation abolishes phase 1, whereas preincubation in the presence of the H^+-ATPase inhibitor bafilomycin abolishes phase 2. Bathing tubules in saline containing bumetanide, an inhibitor of Na^+-$2Cl^-$-K^+ cotransporters, before stimulation abolishes phase 3 but does not alter phases 1 and 2. An increase in apical Cl^- conductance during phase 1 may enhance movement of Cl^- across the apical membrane from cell to lumen, producing the negative going shifts in TEP during this phase. The transepithelial potential reflects contributions of the V-type H^+-ATPase, tending to drive the lumen to more positive values, and compensating movement of Cl^- from cell to lumen, tending to drive the lumen to more negative values. After apical permeability to Cl^- increases in phase 1, the apical membrane potential is strongly influenced by changes in intracellular Cl^- activity following enhanced entry of Cl^- into the cell by activation of a basolateral Na^+-$2Cl^-$-K^+ cotransporter in phase 3. This cotransporter is the dominant mechanism of Cl^- entry. Removal of Na^+ from the bathing saline or addition of bumetanide thus has the same effect on apical membrane potential as does exposure to Cl^- free saline; the lumen rises to a dramatically positive potential, because the H^+-ATPase activity is not compensated by entry of a counterion (825, 1345). Na^+-$2Cl^-$-K^+ cotransport has also been implicated in basolateral entry of ions into Malpighian tubules of most insect species that have been studied (1337).

As noted by Coast (316), the mechanism of ion transport across the secretory tubules of *Rhodnius* is strikingly similar to that seen in tubules of the cricket *Acheta domesticus* (Fig. 12B). Cricket tubules also lack stellate cells and Cl^- is transported across the principal cells and into the lumen via a basolateral Na^+-$2Cl^-$-K^+ cotransporter and apical Cl^- channels. In unstimulated tubules, much of the Na^+ that enters cells via the cotransporter is returned to the bathing medium by a basolateral Na^+/K^+-ATPase (1138), so the secreted fluid is K^+-rich. When the tubules are stimulated with serotonin (*Rhodnius*) or Achdo-DH (*A. domesticus*), the increase in Na^+-$2Cl^-$-K^+ cotransporter activity (321, 825, 1345) overwhelms the activity of the Na^+/K^+-ATPase and the additional Na^+ is exported to the lumen (1138). The Na^+/K^+ ratio of the secreted fluid thus increases from 0.62 to 1.35 in *Rhodnius* (824) and from 0.23 to 1 in *A. domesticus* (316). The higher Na^+/K^+ ratio for tubules of *Rhodnius* may indicate a higher affinity of apical cation/H^+ antiporters for Na^+ (1132). In spite of these close similarities in the apical and basal membrane transporters in tubules of *Rhodnius* and crickets, there are important differences. First, the transport capacities of *Rhodnius* and *A. domesticus* tubules differ enormously when stimulated. Whereas serotonin increases secretion up to 1,000-fold, the increase in response to Achdo-DH is 3- to 4-fold (316). Second, the effects of serotonin on *Rhodnius* tubules are mediated primarily by increases in intracellular cyclic AMP, whereas the effects of Achdo-DH on cricket tubules are mediated, as for other kinins, through increases in intracellular Ca^{2+}.

The apical vacuolar H^+-ATPase The apical vacuolar-type H^+-ATPase is the primary energizer of transport in insect epithelia, and we now have a detailed understanding of its

structure, function and regulation (127). V-ATPases are multisubunit, heteromeric proteins that consist of two structural domains: a peripheral, catalytic V_1 domain ($\sim$ 500 kDa) and a membrane-spanning V_0 domain (100-250 kDa). The stator (consisting mostly of the V_1 domain) anchors the pump to the cell membrane and is responsible for ATP hydrolysis, whereas the rotor (consisting mostly of the V_0 domain) transfers H^+ from one side of the membrane to the other. Immunohistochemical evidence indicates an apical location of the V-ATPase in insect Malpighian tubules, corresponding to the portasomes revealed in transmission electron micrographs (1591, 2074).

An important method of regulating V-ATPase activity is through reversible dissociation of the complex into the V_1 and V_0 domains. In insects, cAMP and PKA are implicated in regulating V-ATPase activity; phosphorylation of subunit C promotes association of V_1 and V_0 and consequent ATP hydrolysis and proton translocation. Other modes of regulation include variation in the coupling ratio between ATP hydrolysis and H^+ translocation, as well as inhibition of V-ATPase activity by reversible formation of a disulphide bond between two cysteine residues in subunit A of the V_1 domain. The most dramatic regulation may be indirect, through alteration of membrane Cl^- permeability. Studies of another secretory epithelium, the blowfly salivary gland, provide ideas as to how this regulation is achieved (78). V-ATPases are inherently electrogenic because their activity pumps protons without an associated anion. In the absence of other charge carriers, therefore, the V-ATPase generates a large voltage across a membrane and produces a modest change in H^+ concentration. When Cl^- permeability is low, as in unstimulated tubule cells, the large lumen-positive potential back-inhibits the activity of the pump. Increased Cl^- permeability (e.g., through inositol trisphosphate-mediated increases in intracellular Ca^{2+}) dissipates the electrical component of the V-ATPase activity, thereby depolarizing the transmembrane potential and allowing pump activity to increase. Thus, when anion channels (e.g., Cl^-) are active, the secretion tends to be more acidic. By contrast, an alkaline secretion is generated in the presence of a cation—nH^+ antiporter or symporter and appropriate counterions (anions).

In contrast to the Na^+/K^+-ATPase and other P-type-ATPases, V-ATPases do not form a phosphorylated intermediate and are therefore relatively insensitive to vanadate, a phosphate-mimic. V-ATPases are highly sensitive to the potent and specific inhibitor bafilomycin A_1, a macrolide antibiotic (452) and to archazolid (817) Fluid secretion by the Malpighian tubules of several species examined is inhibited by 1-10 μM bafilomycin (1337).

Apical K^+/H^+ and Na^+/H^+ exchange The V-ATPase creates both electrical and chemical gradients that can be used to drive the exchange of cellular K^+ (or Na^+) for lumenal H^+ across the apical membrane of insect epithelia. In the midgut of *Manduca sexta*, the exchanger is electrogenic; $2H^+$ are exchanged for each K^+ ion. Exchange is thus driven primarily by the large lumen-positive electrical gradient established across the apical membrane by the V-ATPase (1991). Exchange of $2H^+$ for each K^+ or Na^+ also seems to be required in Malpighian tubules of *Aedes aegypti* (127), as discussed below. By contrast, measurements of membrane potential, intracellular and lumenal pH in Malpighian tubules of ants (2073) and *Rhodnius* (824), show that electroneutral exchange ($1H^+$: $1K^+$ or $1H^+$: Na^+) is sufficient to drive alkali cations from cell to lumen. When *Rhodnius* tubules are stimulated with the diuretic factor 5-HT, intracellular pH acidifies slightly from 6.97 to 6.92 and lumen pH shifts to a more alkaline value, from 6.08 to 6.32 (824). Apical Na^+/H^+ and K^+/H^+ exchange activity may thus be stimulated to a greater extent than the V-ATPase, thus tending to drive the lumen slightly alkaline and the cells slightly acid, relative to unstimulated tubules. Application of amiloride, which blocks Na^+/H^+ exchangers, inhibits fluid secretion and acidifies the lumen, consistent with continued operation of the apical V-ATPase when apical Na^+/H^+ exchange is blocked. The molecular identity of the alkali cation:proton exchangers in the apical membrane has been the subject of several studies using dipteran insects and is therefore discussed in more detail in the section describing tubules with two cell types: principal and stellate.

Na^+-$2Cl^-$-K^+ cotransport Movement of Cl^- from the hemolymph into the cell is thermodynamically uphill, thus ruling out Cl^- entry into the cells through channels (316, 821). Bumetanide-sensitive Na^+-$2Cl^-$-K^+ cotransport is a form of tertiary active transport of Cl^- across the basolateral membrane. The V-ATPase creates the proton motive force that energizes secretion of Na^+ and K^+ into the lumen through the apical cation:proton antiporters. The reduction in cellular Na^+ thus creates a favorable gradient for Na^+ entry across the basolateral membrane. The Na^+ gradient, in turn, energizes Na^+-$2Cl^-$-K^+ cotransport. Measurements with double-barrelled ion-selective microelectrodes show that Cl^- entry via the Na^+-$2Cl^-$-K^+ cotransporter is favored thermodynamically (316, 821) (Fig. 13).

An unusual aspect of the basolateral cation:Cl^- cotransporter in *Rhodnius* Malpighian tubules is that K^+ can be replaced by Na^+. In response to complete removal of K^+ from the bathing saline, 5-HT-stimulated Malpighian tubules secrete fluid at 45% of the rate in the control saline and remain sensitive to bumetanide. Transport is unaltered by the drug hydrochlorothiazide, an inhibitor of Na^+:Cl^- cotransporters in vertebrate tissues. Importantly, dose-response curves relating percentage inhibition of fluid secretion to bumetanide concentration are identical for tubules bathed in control (K^+-replete) and K^+-free saline, suggesting that fluid secretion involves the same bumetanide-sensitive cotransport system in the presence or absence of K^+. Kinetic analysis also supports the idea that Na^+ competes with K^+ for transport by a basolateral bumetanide-sensitive cation/Cl^- cotransporter during fluid secretion (822). *Rhodnius* produces copious amounts of NaCl-rich urine during diuresis,

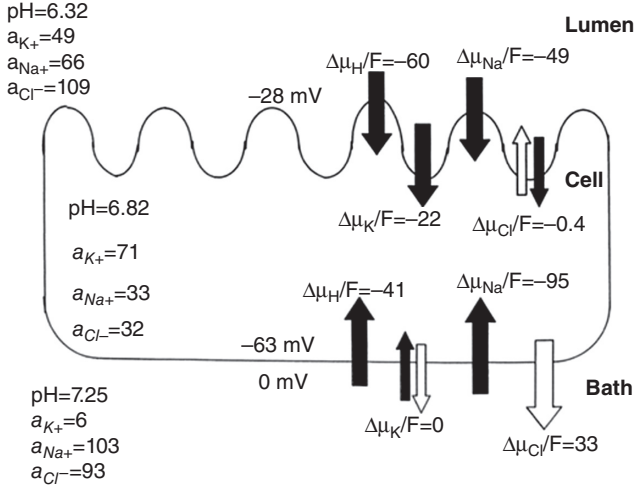

Figure 13 Mean values for electrochemical gradients, ion activities and pH in 5-HT-stimulated Malpighian tubules of *Rhodnius prolixus*. Values of pH and ion activity (a_K, a_{Cl}, a_{Na}) were recorded 30 min after stimulation with 1 μmol l^{-1} 5-HT. Electrochemical gradients ($\Delta\mu/F$, in mV) for Na^+, K^+ and Cl^- across the apical and basolateral membranes are taken from (821, 824).

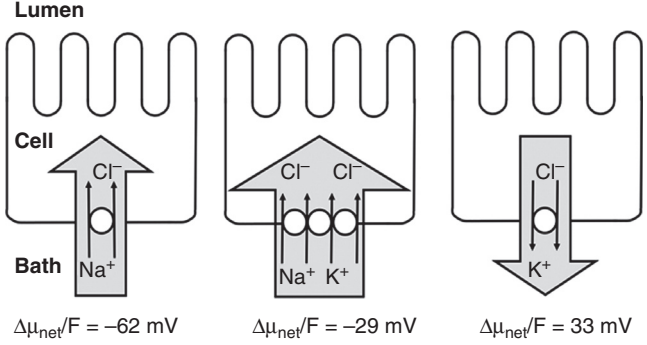

Figure 14 Schematic diagram showing net electrochemical potentials ($\Delta\mu_{net}/F$) for three cation:Cl cotransporters in serotonin-stimulated tubules of *Rhodnius prolixus*. (A) Na^+-$2Cl^-$-K^+, (B) Na^+-Cl^- and (C) K^+-Cl^-. Based on data in (821).

essentially eliminating the nutrient-poor plasma fraction of the blood meal and retaining the nutrient-rich blood cells. Competition between Na^+ and K^+ for transport by a single bumetanide-sensitive cotransporter in the upper tubule provides the means for reducing secreted fluid K^+ concentration, thereby reducing the need for K^+ reabsorption by the lower Malpighian tubule, and at the same time enhancing Na^+ elimination.

Transport of Cl^- across the apical membrane Intracellular chloride activity in *Rhodnius* tubules declines when the apical membrane potential becomes more lumen-positive in Na^+-free saline, K^+-free saline or in the presence of bumetanide (821). This decline suggests the presence of apical Cl^- channels that mediate transport of Cl^- from cell to lumen when entry of Cl^- into the cell across the basolateral membrane is blocked. In Na^+-replete saline, intracellular Cl^- activity is near equilibrium across the apical membrane both in unstimulated and in serotonin-stimulated tubules, as expected if Cl^- channels mediate its transport across the apical membrane. Although fluid secretion by *Rhodnius* tubules is inhibited by 4,4′-diisothiocyano-2, 2′-disulphonic acid stilbene (DIDS), transepithelial potential and intracellular pH are unaltered, as would be expected if DIDS blocked apical Cl^- channels or Cl^-/HCO_3^- exchangers, respectively. Cl^- channels, if present, are DIDS-insensitive, and the effects of fluid DIDS on fluid secretion may reflect indirect effects of DIDS, such as inhibition of mitochondrial functioning.

Other pathways for Cl^- movement, such as apical K^+:Cl^- cotransport proposed for *Aedes* tubules (see below) and/or paracellular Cl^- movement, can be ruled out because they are thermodynamically unfeasible. The calculated electrochemical gradients indicate that a K^+:Cl^- cotransporter in the apical membrane of *Rhodnius* Malpighian tubules would mediate net movement of these ions from the lumen into the cell, that is, in the opposite direction to that required for fluid secretion (Fig. 14). Although the transepithelial potential across tubules of most insects is lumen-positive, the TEP in fully stimulated *Rhodnius* tubules is lumen-negative, and a passive paracellular pathway for Cl^- movement is thus precluded.

K^+ channels Although the K^+ channel blocker Ba^{2+} alters the basolateral membrane potential in tubules of *Rhodnius* or *Acheta domestica,* it has no effect on fluid secretion (316). It thus appears that K^+ channels are not implicated in net transepithelial K^+ and fluid secretion by Malpighian tubules of these two species. By contrast, multiple mechanisms of K^+ transport, including channels, may be necessary in tubules of the ant, *Formica polyctena* because hemolymph K^+ levels are quite variable. The model of Leyssens et al. (1044) proposes that a Na^+-$2Cl^-$-K^+ cotransporter that is sensitive to 10^{-5} M bumetanide is the primary mechanism of uptake at the lowest bathing saline K^+ concentrations of 5 to 10 mM. At the intermediate concentration of 51 mM K^+, a K^+:Cl^- cotransporter sensitive to high (10^{-4} M) concentrations of bumetanide becomes active. At very high bathing saline concentrations (113 mM K^+), K^+ enters through basolateral Ba^{2+}-sensitive K^+ channels (1044).

Basolateral K^+ channels may also contribute to tubule K^+ secretion in the black field cricket *Teleogryllus oceanicus* (2048). As in the ant *F. polyctena*, hemolymph K^+ and Na^+ probably vary as a result of omnivory, and K^+ may enter the Malpighian tubule cells through Ba^{2+}-sensitive K^+ channels under some conditions. At lower hemolymph K^+ concentrations, bumetanide inhibits fluid secretion by 80% and inhibits Na^+, K^+ and Cl^- secretion by 60%, 70% and 50%, respectively. This result suggests that a large proportion of net transepithelial ion transport is mediated by cotransport of these three ions (2048).

Malpighian tubules composed of principal cells and stellate cells

Malpighian tubules in which the secretory segment is comprised of two structurally distinct cells known as principal cells and secondary or stellate cells have been studied most extensively in the mosquito *Aedes aegypti* and the fruit fly *Drosophila melanogaster*. The model of transport that has emerged from studies over the last decade emphasizes the role of the stellate cells in Cl^- secretion and the principal cells in secretion of Na^+ and K^+ (Fig. 15). For both cations and anions, the primary energizer of transport is the V-type H^+-ATPase on the apical (lumen-facing) membrane of the principal cells. The electrogenic nature of the H^+ pump means that both electrical and chemical gradients are used to drive the transport of Na^+, K^+ and Cl^-. For Na^+ and K^+, apical cation:H^+ exchangers of the cation:proton antiporter (CPA) family allow lumenal H^+ to leak from the lumen back into the cell in exchange for Na^+ or K^+ (1535, 2046). In bacteria, related cation:proton antiporters in the Kef family are electrogenic; for the tubules, a stoichiometry of $2H^+$:$1K^+$ or $2H^+$:$1K^+$ would allow cation secretion to be driven, in part, by the lumen-positive potential created by the H^+-ATPase. Microarray data and in situ analyses show that *Drosophila* genes CG10806 and CG31052 are members of the CPA2 family and are homologues of bacterial Kef exchangers. Immunocytochemistry localizes endogenous CG10806 and CG31052 to the apical plasma membrane of the Malpighian (renal) tubule (400).

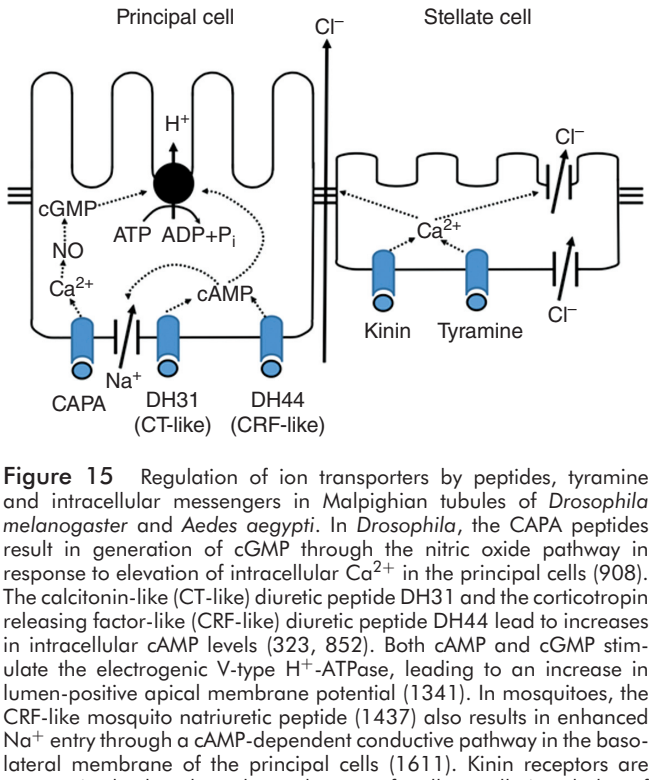

Figure 15 Regulation of ion transporters by peptides, tyramine and intracellular messengers in Malpighian tubules of *Drosophila melanogaster* and *Aedes aegypti*. In *Drosophila*, the CAPA peptides result in generation of cGMP through the nitric oxide pathway in response to elevation of intracellular Ca^{2+} in the principal cells (908). The calcitonin-like (CT-like) diuretic peptide DH31 and the corticotropin releasing factor-like (CRF-like) diuretic peptide DH44 lead to increases in intracellular cAMP levels (323, 852). Both cAMP and cGMP stimulate the electrogenic V-type H^+-ATPase, leading to an increase in lumen-positive apical membrane potential (1341). In mosquitoes, the CRF-like mosquito natriuretic peptide (1437) also results in enhanced Na^+ entry through a cAMP-dependent conductive pathway in the basolateral membrane of the principal cells (1611). Kinin receptors are present in the basolateral membranes of stellate cells in tubules of both *Aedes* and *Drosophila* (1105, 1495). Both the kinins (125, 1495, 1496, 1817) and tyramine (146-148) result in increases in intracellular Ca^{2+}. In *Drosophila*, Ca^{2+} is thought to stimulate an increase in transcellular chloride permeability through actions on apical Cl^- channels. In *Aedes*, Ca^{2+} is thought to increase the permeability of the paracellular pathway (126).

Entry of Na^+ and K^+ into the cells across the basolateral membrane involves multiple transporters. Na^+ may exchange for H^+ through NHE3; the reaction may also contribute to intracellular pH regulation. Bumetanide-sensitive cotransport of Na^+-$2Cl^-$-K^+ has been shown to be important for tubules of both *Aedes* (725, 1641) and *Drosophila* (823). In addition, a role for the Na^+/K^+ ATPase is seen in the inhibitory effects of ouabain on fluid secretion by tubules of both mosquitoes (725) and *Drosophila* (1074, 1844). Immunocytochemical evidence has demonstrated the presence of the Na^+/K^+-ATPase in the principal cells of the adult tubule in *Aedes* (1407).

Intracellular microelectrode recordings from principal cells reveal that ouabain depolarizes the basolateral membrane of the principal cells by $\sim$ 8 mV from the control value of ~ -60 mV (1074). The Na^+/K^+-ATPase may thus play an ancillary role in tubule secretion; its stoichiometry of $3Na^+$:$2K^+$ means that it may enhance basolateral entry of K^+ at the expense of Na^+; inhibition of secretion with ouabain is associated with an increase in secreted fluid Na^+ concentration of approxiimately 50% (1074), as seen previously in unstimulated tubules of *Rhodnius* (1138). Much of the Na^+ that enters the cell through the Na^+-$2Cl^-$-K^+ cotransporter is recycled from cell to hemolymph by the Na^+:K^+-ATPase, contributing to secretion of K-rich fluids (120 mM K^+: 30 mM Na^+; [823]) by *Drosophila* tubules. The contribution of the Na^+/K^+-ATPase has been underestimated because transport of ouabain by an organic anion-transporting polypeptide reduces the effective concentration of the inhibitor (1844). This had lead to the erroneous conclusion that the Na^+/K^+ ATPase was not present in insect Malpighian tubules. The Na^+/K^+-ATPase can be viewed as an ancillary transporter whose roles are most apparent in tubules secreting at the basal rate. Under such conditions, it can play an important role in setting the Na^+:K^+ ratio of the secreted fluid and in maintaining the Na^+ gradient across the basolateral membrane for Na^+-coupled transporters such as those implicated in elimination of small hydrophilic organic anions. When tubules respond to diuretic factors, the apical H^+-ATPase is stimulated and plays a dominant role in transepithelial secretion.

Chloride ions that enter through the Na^+-$2Cl^-$-K^+ cotransporter may exit the cell into the lumen through apical Cl^- channels. These may be in the principal cells, as is the case for insects with only a single cell type in the tubules, or Cl^- may exit through Cl^- channels in the apical membrane of the stellate cells (1329, 1347). Although dense accumulations of Cl^- channels have been identified in stellate cells of *Aedes* tubules, the latter pathway would require gap junctions between the principal cells and stellate cells (126). Alternatively, transepithelial secretion of Cl^- may involve basolateral entry into the principal cells through a Cl^-/HCO_3^- exchanger (1453).

Measurements with double-barrelled ion-selective microelectrodes reveal that the electrochemical potential favors passive movement of K^+ from cell to bath for the principal cells of *Drosophila melanogaster* (823). A direct contribution of K^+ channels to transepithelial fluid secretion can thus be ruled out. Similar results were reported in unstimulated Malpighian tubules of *Rhodnius prolixus* (821). K^+ channels may play a role in transepithelial ion transport in tubules of other species if intracellular K^+ activity is below electrochemical equilibrium across the basolateral membrane, as proposed for Malpighian tubule cells of the ant *Formica polyctena* (1045) and the weta *Hemideina maori* (1305).

Reabsorption of Water and Ions

Most absorption of nutrients and water occurs across the midgut in insects. The hindgut then serves to modify what it receives from two upstream sources: the gut contents following digestion and absorption across the midgut as well as the fluid secreted by Malpighian tubules. Although the hindgut performs the bulk of reabsorption of useful ions and water in most species, a proximal (lower) segment of the Malpighian tubule modifies the urine of some species. A similar role is performed by the ileum of the locust, *Schistocerca gregaria* (1444). Such reabsorption upstream allows the rectum to process more thoroughly a reduced volume of fluid delivered to it.

Urine modification by the lower (proximal) segments of the Malpighian tubules

There appear to be two reasons for reabsorption of water and/or ions by what is known as the lower or proximal tubule (i.e., that portion of the tubule's length closest to the junction with the gut). For a blood feeder such as *Rhodnius prolixus*, the high surface area offered by the lower Malpighian tubule relative to the volume of fluid in its lumen permits more rapid reabsorption of KCl during the post-prandial diuresis than would be possible in the large, sac-like rectum. In *Drosophila* and other dipterans, there is extensive reabsorption across the rectal pads of the hindgut, and the lower Malpighian tubule probably serves a preparatory or ancillary role in the reabsorption of both K^+ and water.

Reabsorption of ions but not water by the *Rhodnius* tubule is a consequence of the insect feeding on blood, which is hypoosmotic to its hemolymph. Homeostasis requires elimination of hypoosmotic urine that is produced in a two stage process: production of near-isoosmotic primary urine (consisting of approximately 100 mM NaCl, 80 mM KCl) by the upper secretory segment of the Malpighian tubule, followed by reabsorption of ions but not water as the secreted fluid passes through the 30% of the lower tubule's length closest to the hindgut (1129). Water is not reabsorbed because the osmotic permeability of the lower tubule is much lower in this region relative to the upper tubule or the upper region of the lower tubule (1331). KCl reabsorption by the lower tubule preserves osmotic and ionic homeostasis; the fluid passing into the hindgut is both NaCl-rich and hypoosmotic.

The current model of KCl reabsorption suggests that K^+ is first pumped thermodynamically uphill from lumen to cell by an ATP-dependent transporter that is inhibited by drugs such as omeprazole, which block the H^+/K^+-ATPase of the vertebrate gastric mucosa. K^+ then moves passively from cell to bathing saline (hemolymph) through an electrodiffusive pathway (i.e., channels) that can be blocked by Ba^{2+}. The presence of basolateral K^+ channels is indicated by changes in basolateral membrane potential in response to changes in bathing saline K^+ concentration and blockade of these changes and of K^+ reabsorption by Ba^{2+}. K^+ reabsorption is unaffected by amiloride (which blocks a putative K^+/H^+ exchanger in the upper tubule) and by the V-type-ATPase inhibitor bafilomycin A_1, applied to the basolateral or apical surfaces of the lower tubule (685).

Chloride also moves from cell to hemolymph through channels. Chloride channel blockers such as diphenylamine-2-carboxylate and the related compound 5-nitro-2-(3-phenylpropylamino) benzoic acid inhibit KCl reabsorption and also block Cl^--dependent changes in basolateral membrane potential (686). KCl reabsorption is also reduced by carbonic anhydrase inhibitors such as acetazolamide, consistent with a role for cellular CO_2 as a source of both H^+ and HCO_3^- for exchange of K^+ and Cl^-, respectively. The mechanism of Cl^- transport from the tubule lumen into the cell is unclear, although a stilbene-insensitive Cl^-/HCO_3^- exchanger has been proposed.

It is worth noting that the lower tubule of adult *Drosophila melanogaster* performs multiple functions; it reabsorbs approximately 30% of the KCl and water secreted upstream by the main segment of the tubule. In addition, the lower tubule secretes Ca^{2+} from the hemolymph into the lumen, acidifies the lumenal fluid and also secretes organic cations such as tetraethylammonium at high rates (1344, 1534). In larvae, the lower tubule secretes K^+ but reabsorbs Na^+ (1287). The lower Malpighian tubule is thus much more than a simple conduit connecting the fluid secretory main segment to the ureter and gut. Fluid secreted upstream is also modified by reabsorptive process in the lower tubule, the ampulla (into which multiple tubules empty and which is itself connected to the gut), or both in the house cricket *Acheta domesticus* (1745). There are also reabsorptive cells scattered throughout the epithelia in tubules of the cricket *Teleogryllus oceanicus* (1160).

The Insect Hindgut

The hindgut receives the output of the midgut and the Malpighian tubules; the contents of the hindgut are then modified through reabsorption of useful solutes and water and elimination of molecules that are present in excess or are toxic. The hindgut—and the rectum in particular—is thus a major site of water conservation in insects. An extreme example is evident in the rectum of the mealworm larvae (*Tenebrio*

molitor); water reabsorption is so effective that the fecal pellets that are expelled are almost completely dry. In the hindgut of stick insects (1501, 1502), water-stressed locusts and cockroaches (1441, 1442, 1924), as much as 80%-95% of the ions and water secreted by the Malpighian tubules are reabsorbed; the corresponding figure for blowflies is 65%. When necessary, the extent of water reabsorption by the insect hindgut can be reduced. When locusts are fed on a succulent diet, for example, less than 10% of water is reabsorbed from the hindgut (1099), and blood-feeders such as *Rhodnius* recover virtually no water during diuresis.

There appear to be at least four distinct physiological mechanisms of water conservation in the hindgut of different species of insect (168, 1439).

Cockroaches, locusts and adult blowflies—absorption of hypoosmotic fluid by rectal pads or papillae

In these species there are conspicuous thickenings of the rectal epithelium that are referred to as rectal pads in locusts and cockroaches and as rectal papillae in adult flies. The processes of local osmosis and solute recycling during fluid absorption by the rectal pads of cockroaches were demonstrated in classic work by Wall (1924). Osmotic gradients for absorption of water from the rectal lumen are produced though active transport of Na^+, K^+ and Cl^-. Fluid and ions move from the rectal lumen into the epithelial cells of the pads and then into the intercellular spaces between the epithelial cells. The intercellular spaces thus act as a separate compartment from the hemolymph and the rectal lumen. Salt transport elevates the osmotic concentration in the intercellular spaces above that in the lumen, so that water enters by osmosis, distending the intercellular spaces. Downstream, ions are reabsorbed into the epithelial cells as the solutes and water move along the intercellular channels. A high flow rate and relatively low osmotic permeability are presumed to minimize water movement back into the cells, so that a hypoosmotic fluid is transferred to the hemolymph. The ions reabsorbed into the cytoplasm can then be recycled back into the upstream sections of the intercellular spaces so as to permit further osmotic reabsorption from the lumen. Microsampling of fluid from the rectal tissues shows that fluid in the lateral intercellular spaces near the lumen is hyperosmotic, and the total ion concentration in the intercellular channel declines as fluid moves toward the hemocoel.

The main driver of ion transport in the locust rectum is distinct from the Na^+/K^+-ATPase, which dominates vertebrate epithelial ion transport and the vacuolar type H^+-ATPase, which plays a cardinal role in other insect epithelia such as the midgut and Malpighian tubules. Instead, a primary mechanism of Cl^- transport is responsible for absorption of ions and water. Exhaustive and elegant work by John Phillips and coworkers has shown that Cl^- transport is not coupled to, or driven secondarily by, movements of Na^+, K^+, HCO_3^-, Ca^{2+} or Mg^{2+} (1439, 1444). An apical V-type H^+-ATPase is present and it acidifies the hindgut lumen, but its rate of transport is only 10%-15% of Cl^--dependent short-circuit current across the rectum. Regulation of rectal Cl^- transport is accomplished by two stimulatory factors that act through intracellular cyclic AMP: chloride transport stimulating hormone (CTSH) and ion transport peptide (ITP), as described below.

Mosquito larvae—secretion of hyperosmotic salt solutions by rectal salt glands

Hemolymph osmolality in the larvae of saline-tolerant mosquitoes is ~300 mosmol/kg, more than three times lower than that in the surrounding medium. Loss of water by osmosis across the body surface is compensated by drinking the medium, but the ingested salt must then be eliminated. Whereas ions and useful metabolites are reabsorbed across the anterior segment of the rectum (169, 1784), saltwater mosquito larvae eliminate excess salt by active secretion of Na^+, K^+, Mg^{2+} and Cl^- across the posterior rectum from the hemolymph into the lumen (171).

Tenebrionid beetle larvae—secretion of hyperosmotic salt solutions within the cryptonephridial complexes

Survival of larvae of beetles such *Tenebrio molitor* in extremely dry conditions depends on effective reabsorption of water from the rectum before the fecal pellets are eliminated. The structure responsible for water reabsorption is the cryptonephridial complex, which is comprised of the distal portions of the Malpighian tubules applied closely to the surface of the rectum by an enveloping perinephric membrane. The rectum and the tubules thus act in concert. K^+, Cl^- and to a lesser extent Na^+ are actively secreted from the hemolymph into the lumen of each of the six Malpighian tubules. In some tenebrionid larvae, the concentration of KCl in the tubule lumen approaches and may even transiently exceed saturation. This "osmotic sink" within the tubule lumen (1121) is used both for fecal dehydration and for atmospheric water vapor absorption. The rectal epidermis plays a passive role, providing osmotic coupling between the rectal lumen and the tubule lumen. The function of the perinephric membrane is to provide an osmotic barrier to limit osmotic water flow from the hemolymph into the complex in response to the elevated osmolality within the Malpighian tubule lumen; instead, water is extracted from the rectal lumen. Beneath the perinephric membrane and surrounding the tubules is the perinephric space. The activity of K^+ in the perinephric space is close to electrochemical equilibrium with the hemolymph, whereas the activities of Na^+ and H^+ are reduced fivefold and threefold, respectively, below the corresponding Nernst equilibrium values. These data are consistent with a model in which cations move from the hemolymph across the perinephric membrane into the perinephric space, which is the proximal source for cations transported into the tubule cells

and then the lumen (1332). In millipedes, it appears that a cryptonephric arrangement used for fecal dehydration and water vapor absorption has evolved independently from that of the tenebrionids (2038).

Thysanurans (silverfish and firebrats) and flea larvae—rectal and anal sacs

In thysanurans, there is no evidence for high concentrations of inorganic salts in the structures implicated in fecal dehydration and water vapor absorption (1333). The anal sac consists of a highly folded single-layered epithelium. The subcuticular space between the rectal cuticle and the epithelium is filled with a material that resembles glycerol and also contains mucopolysaccharides. The necessary osmotic gradient to drive water reabsorption from the lumen appears to involve dehydration of this material by transport activity of the epithelial cells. Water movement may be produced by electroosmosis (984). In this proposal, a lumen-positive potential of 200 mV is generated by active transport of K^+ across the apical membrane of the rectal epithelial cells. It is suggested that cations are then recycled into the cells through cation-selective channels, and that water is coupled electroosmotically to the inward current. Water thus moves first from the rectal lumen onto the hygroscopic material of the subcuticular space and subsequently into the cytoplasm of the epithelial cells. The weakness of this proposal is that coupling of ions to water occurs in the subcuticular space at water activities that are well below the saturation point of KCl (1333).

In flea larvae, a region of the rectum called the rectal sac has been implicated in reduction of water activity in the recta lumen during water vapor absorption (114). The epithelial cells of the rectal sac are arranged in a ventral and a dorsal gutter and show a distinct asymmetry along the length of the organ. Dorsal and ventral gutter epithelial cells show large numbers of mitochondria associated with deep membrane infoldings, but whereas the apical membrane is infolded in the dorsal cells, the basolateral membrane is infolded in the ventral cells. As for the thysanurans, there is no evidence for high solute concentrations that might be used to produce colligative lowering of vapor pressure to drive faecal dehydration and water vapor absorption. The threshold humidity at which net vapor absorption is feasible is 65% RH, well below the vapor pressure over saturated solutions of NaCl (75%) and KCl (85%).

Anal Papillae

The larvae of mosquitoes, midges and fruit flies possess extrarenal organs known as anal papillae that have been implicated in hemolymph ion regulation and osmoregulation. The papillae are produced during embryonic development, by eversion of the hindgut tissues. In mosquito larvae, four anal papillae arise from an extension of the terminal segment and project into the external medium. The walls of the papillae are composed of a one cell thick epithelial syncytium and the lumen is continuous with the hemolymph. The apical surface of the papilla is separated from the external medium by a thin external cuticle. The function of the anal papillae is uptake of ions, primarily Na^+ and Cl^-, from the external medium (442, 1771-1773), thereby contributing to the maintenance of proper hemolymph ion levels.

In common with other epithelial cells implicated in salt and water transport, the apical and basal plasma membranes of the cells of the papillae have many infoldings that are closely associated with mitochondria (588, 1725). Both morphological and ultrastructural characteristics are consistent with a role for the papillae in ion uptake from dilute media. The papillae become longer when larvae are reared in very dilute water. Conversely, the apical and basal membrane infoldings decrease and there are fewer mitochondria in the papillae of larval *Aedes aegypti* raised in saltwater. Anal papillae of mosquito larvae also show short term changes in transport characteristics when the environmental salinity is altered; Na^+ and Cl^- uptake decrease in higher salinity (441).

The mechanism of transport across the papillae shows some similarities to the gills of freshwater fish. In larvae of the midge *Chironomus riparius*, inhibitors of the Na^+/H^+ exchangers (EIPA) and carbonic anhydrase (methazolamide) provide evidence for Na^+/H^+ and Cl^-/HCO_3^- exchange mechanisms in the anal papillae. Comparable studies of the papillae of *Aedes aegypti* suggest that Na^+ uptake at the apical membrane occurs through a Na^+ channel that is driven by a V-type H^+-ATPase, or through a Na^+/H^+ exchanger that is insensitive to amiloride and its derivatives (415). Cl^- uptake occurs through a Cl^-/HCO_3^- exchanger, with carbonic anhydrase providing H^+ to the V-type H^+-ATPase and HCO_3^- to the exchanger. It is suggested that Cl^- subsequently crosses the basal membrane into the lumen of the papilla through channels, whereas Na^+ may be pumped from cell to lumen via the Na^+/K^+-ATPase. Electron micrographs reveal that the apical plasma membrane is studded with portasomes, consistent with the presence of the V-ATPase. Immunohistochemical studies indicate the presence of an apical V-ATPase and a basal Na^+/K^+-ATPase. Transcripts of putative aquaporin genes are expressed in the anal papillae, which also show immunoreactivity to a human AQP1-antibody. In conjunction with observed inhibition of tritiated water uptake in isolated anal papillae by the aquaporin blocker mercury, it thus appears that aquaporins faciliate water transport across the cellular membranes of the papillae (1179).

Neuroendocrine Control of the Malpighian Tubules and Gut

Most insects spend much of their lives in a state of antidiuresis given that they live in a desiccating environment. Thus, although the normal condition involves elimination of relatively dry excreta, excess water is voided after eclosion

and also in response to feeding on moist or liquid diets. Diuretic hormones act in most species by accelerating the production of the primary urine by the Malpighian tubules. Antidiuretic hormones act inhibiting fluid production by the tubules or, more commonly, by stimulating reabsorption across the hindgut (1746). The initially paradoxical discovery of diuretic hormones in desert insects has been explained by noting that clearing the hemolymph of wastes and toxins requires stimulation of fluid secretion by the Malpighian tubules followed by downstream reabsorption of water in the hindgut. Factors that stimulate this process are thus better described as clearance hormones (1306).

One rationale for studying neuroendocrine control mechanisms for osmoregulatory processes in insects is that such studies offer potential for development of novel, species-specific and environmentally benign control measures for pest species. Insect kinin neuropeptides, for example, have been isolated from many species and have been implicated in control of processes such as hindgut contraction, diuresis and the release of digestive enzymes. Biostable analogs have been designed so as to protect sites in the pentapeptide kinin sequence from peptidases in the hemolymph and tissues of insects. In particular, peptide analogs containing an aldehyde at the C terminus are known to inhibit various classes of proteolytic enzymes. C-terminal aldehyde insect kinin analogs lead to fluid retention and mortality in corn earworm larvae and also interfere with diuresis in the housefly (1268). Peptidomimetic analogs that contain no native peptide bonds but retain significant diuretic activity in an *in vitro* cricket Malpighian tubule fluid secretion assay have also been developed (1267).

The Malpighian Tubules

Factors that alter fluid and ion transport by MTs include biogenic amines (serotonin and tyramine) and five families of neuropeptides (315, 317, 319, 321). Among the best-studied peptides are the corticotrophin-releasing factor-related diuretic hormones (CRF-related DHs) and the kinins, so named for their effects on hindgut contractions. A third peptide family is made up of the calcitonin-like diuretic hormones (CT-like DH). In addition, tubules of locusts and the hawkmoth *Manduca sexta* are stimulated by tachykinin-related peptides. The CAPA peptides, products of the capability (*capa*) gene in *Drosophila*, are unusual in that they exert diuretic or antidiuretic effects on tubules of different species. In dipterans, CAPA peptides elevate production of intracellular nitric oxide, which, in turn, activates guanylate cyclase (201, 397, 448, 1125). The increase in cGMP is associated with stimulation of the V-type H^+-ATPase in the apical membrane and a consequent increase in transport of K^+ and/or Na^+ across the apical membrane (1347). The increase in ion transport and the flow of osmotically obliged water results in the increased fluid flow is seen during diuresis. By contrast, CAPA peptides act as antidiuretics in hemipteran tubules, including the blood-feeder *Rhodnius prolixus* (1385, 1392-1395) and the plant-sucking bugs *Acrosternum hilare* and *Nezara viridula* (322). Antidiuretic factors of the beetle *Tenebrio molitor* (Tenmo ADFa and Tenmo ADFb) also lead to increases in cGMP levels, but do so through mechanisms that are independent of nitric oxide signaling, and so a soluble guanylate cyclase is unlikely (485, 486, 1993). Tenmo ADFa and cGMP also inhibit fluid secretion in tubules of adult *Aedes aegypti*; the inhibition by ADF does not involve changes in transepithelial voltage or resistance, suggesting that electroneutral transport is affected, possibly the basolateral Na^+-$2Cl^-$-K^+ cotransporter in the principal cells (1180).

It has been a long-standing question as to why such a multiplicity of factors that alter the Malpighian tubules is needed. One possibility is that the use of two factors allows for a synergistic interaction. The net effect of such an interaction is to steepen the dose-response curve to the point that the two factors together act essentially as a switch, converting an unstimulated tubule into a fully diuretic one. Locustakinin (Locmi-K) and Locusta-diuretic hormone (Locmi-DH), for example, act synergistically to stimulate secretion by Malpighian tubules of the desert locust *Schistocerca gregaria*. When both are present, fluid secretion is stimulated to a greater extent than the sum of their individual effects. Kinin effects are mediated through increases in intracellular inositol trisphophate/Ca^{2+}, whereas those of Locmi DH are mediated by increases in cAMP. Such synergism would allow the use of lower concentrations of diuretic factors to be released, thus minimizing the energetic costs associated with their synthesis and release. Alternatively, some factors may be implicated in excretion of specific ions (Na^+ versus K^+) or in integrating various other epithelia (salivary glands, midgut, hindgut). Locmi-DH, for example, increases Na^+ transport by locust tubules at the expense of K^+ transport in the presence or absence of locustakinin. Enhanced Na^+ transport is also seen when MTs are stimulated with cAMP. Increased Na^+ secretion by Lom-DP acting alone or in synergism with locustakinin may serve to provide sufficient Na^+ for use by Na^+-coupled cotransporters downstream in the tubule or hindgut (318). Independent control of Na^+ and K^+ secretion may be important, for example, in maintenance of hemolymph volume and composition after feeding. For the Malpighian tubules of the tobacco hawk moth *Manduca sexta*, there are at least eight different sorts of compounds that significantly alter the rates of Malpighian tubule fluid secretion, and all of these factors also accelerate the rate of heartbeat in *Manduca* and do so at similar concentrations (1707). To quote the authors of the latter study, "[T]here may exist in the extracellular fluid a continuous broadcast of information in the form of a chemical language, to which many or all parts of the body continuously respond on a moment-to-moment basis and which, because of the greater information in it, ensures a more effective and efficient coordination of function than could be achieved by a series of single, tissue-specific hormones that force stereotypical responses by their target tissue."

Hindgut and Midgut

Most of what we know of hormonal control of fluid reabsorption by the insect hindgut is based on studies of locusts, in part because the large size of the hindgut permits flat sheets of epithelia to be isolated for use in Ussing chambers. Three antidiuretic peptides have been isolated from the corpora cardiaca, a neurosecretory organ analogous to the pituitary and hypothalamus glands of the vertebrate endocrine system: neuroparsins (Np), ion transport peptide (ITP) and chloride transport stimulating hormone (CTSH) (1638). Stimulation of active absorption of Cl^- at the apical membrane by CTSH or ITP involves cAMP as second messenger.

In locusts, hormonal control of the Malpighian tubules and the hindgut are clearly separated. Ion-transport peptide (ITP) released into the hemolymph from the corpora cardiaca stimulates fluid reabsorption by the ileum but has no effect on fluid secretion by the Malpighian tubules. Conversely, locustakinin (Lom-K) or Locusta-diuretic hormone (Locusta-DH) stimulate fluid secretion by locust Malpighian tubules but have no effect on active transport of Cl^- or the rate of fluid reabsorption across the ileum or rectum *in vitro* (320). Responses of the tubules to diuretic factors are more rapid than the response of the ileum and rectum to hindgut stimulants such as ITP. As a consequence, urine flow exceeds fluid uptake in the hindgut in the early phases of post-prandial diuresis, so excess water is eliminated.

Many of the neuropeptides present in the neurosecretory cells of the central nervous system have also been found in nerve cells within the gut or in midgut endocrine cells (2077). These findings raise the likelihood that processes such as absorption of ions and water across the midgut may be modulated by neuropeptides acting as hormones or paracrine factors. Given that secretion of water and ions by the Malpighian tubules of *Drosophila* is stimulated by leucokinins and DH31, it is of interest that receptors for the leucokinins are present in the midgut of the *Drosophila* larvae and receptors for the diuretic hormone DH31 are present in the midgut of both larvae and adults (1907, 1908). Although stimulation of midgut ion transport as part of a diuretic strategy has not been observed in *Drosophila*, serotonin is well known for its role in stimulation of both tubule secretion and midgut Na^+ and water absorption in *Rhodnius* (517, 1136), and the CAPA peptides inhibit both secretion by the tubules and absorption by the midgut (826, 1391). Although high rates of midgut ion transport are seen during feeding by larvae of the tobacco hornworm, there is an abrupt decline in midgut ion transport when feeding ceases and the larvae enter the wandering stage, during which it becomes committed to pupation. Possible hormonal control of this decline in midgut absorption is suggested by the finding that active ion transport across the posterior midgut of the tobacco hornworm is inhibited by *Manduca* allatotropin (Mas-AT) and by two *Manduca* FLRFamides known as F7D (DPSFLRF-NH_2) and F7G (GNSFLRF-NH_2) (1027).

Excretion of Toxins

Insects have an extraordinary capacity to detoxify and excrete an enormous range of potentially toxic molecules. This facility reflects, in part, the coevolution of flowering plants and insects. To minimize herbivory, plants have evolved a wide range of secondary compounds that are toxic. The presence of such compounds in the insect diet has lead to selection for genes that confer some form of resistance to the toxic molecule. The larva of the tobacco hornworm (*Manduca sexta*) can thus survive on a diet containing large quantities of the cholinergic receptor blocker nicotine, and milkweed bugs can feed on plants containing high levels of cardiac glycosides, which are potent inhibitors of the Na^+/K^+-ATPase. This evolutionary arms race between plants and insects has produced a series of "leapfrog" events, whereby appearance of a new toxin in a plant's chemical armoury has led to selection in insects for new enzymes or membrane transporters that can detoxify or excrete the toxin.

Toxin excretion is accomplished by the Malpighian tubules and the gut, including the midgut and hindgut. In the midgut, toxin transporters may play a dual role, minimizing passive uptake of toxins by actively secreting them back into the gut lumen, or in clearing the hemolymph of toxins. Exposure of insects to plant-derived or anthropogenic toxins is also associated with increases in the activity of phase I and phase II detoxification mechanisms. Phase I enzymes introduce reactive and polar groups into substrates by oxidation, hydrolysis or reduction. Phase I enzymes include the P450 monoxygenases, which have been implicated to play a role in metabolism of natural and synthetic pesticides by insects (529). Metabolites of xenobiotics after phase I reactions may be subsequently conjugated with compounds such as glutathione, sulphate or glucuronate in phase II reactions. Glutathione-S-transferases (GSTs) are prominent among the phase II enzymes; increases in GST levels are correlated with resistance to all major classes of insecticides (1511). The phase I and phase II enzymes are found in tissues such as the fat body and also in the Malpighian tubules. Treatments that increase P450 and GST gene expression level are also associated both with increases in expression of toxin transporter genes in the tubules of *Drosophila* and with toxin secretion by isolated tubules. Exposure of *Drosophila* to toxins thus evokes a coordinated response by the Malpighian tubules, involving both alterations in detoxification pathways as well as enhanced transport (266) (Fig. 16).

The hindgut, along with the Malpighian tubules, plays a primary role in excretion of nitrogenous wastes such as ammonia (as described below in the section on Nitrogen Excretion) and uric acid. In desert locusts, ammonium urate is excreted: the urate by the Malpighian tubules and ammonium by both the tubules and the rectum. This arrangement goes against the usual view of uricotely in insects but provides multiple advantages. Ammonium urate has a lower solubility than other urate salts have, so less water is lost when it is excreted, and it also

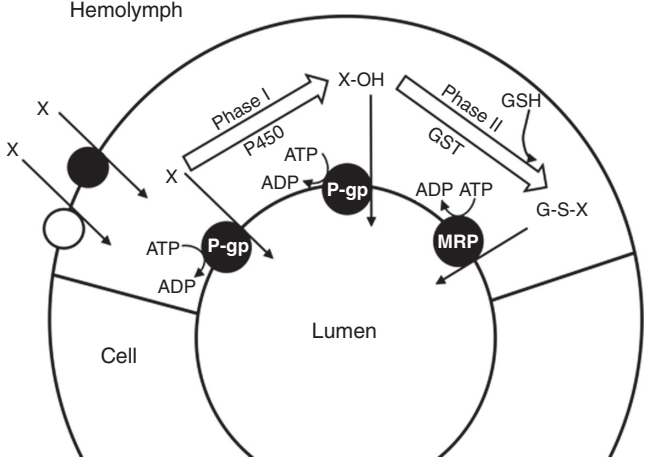

Figure 16 Speculative model showing interaction of detoxification and excretion pathways in Malpighian tubules and gut. A moderately hydrophobic toxin (X) may be transported unmodified into the lumen by apical P-glycoprotein (P-gp) and/or multidrug resistance-associated protein (MRP). Alternatively, the toxin may be modified by P450 enzymes and either transported into the lumen by P-gp, or further modified by conjugation with glutathione (GSH) through the actions of GST and then transported into the lumen by MRP. Toxin X may enter across the basolateral membrane through active (closed circle) or passive (open circle) mechanisms. Adapted from Bard (71, 1337).

has the additional advantage of providing for elimination of one additional nitrogen. Exchange of Na^+ for NH_4^+ in the rectum conserves Na^+, which is in short supply in a herbivorous diet. Moreover, ammonium secretion provides an effective means of increasing acid elimination without increasing the pH gradient against which the hindgut proton pump must work. Proton secretion is implicated in recovery of HCO_3^- in the hindgut and possibly in reabsorption of proline (1440).

Transport of organic cations and organic anions

Both the gut and the Malpighian tubules transport a wide variety of organic ions. Organic ions may be derived from the diet or metabolism, and many are toxic, thus necessitating elimination from the insect. For both organic cations (OCs) and organic anions (OAs), there are multiple transporters, often with overlapping substrate affinities. Most organic cations (OCs) carry a positive charge at physiological pH and contain a hydrophobic region and usually a quaternary nitrogen. The need for multiple transporters is related, in part, to the need to transport molecules from two broad structural classes. Type I organic cations are hydrophilic, monovalent and relatively small (< 400 Da). Examples are endogenous compounds such as choline and N-methyl nicotinamide (NMN) and drugs such as tetraethylammonium (TEA) and quinine. Type II organic cations are larger (> 500 Da), amphiphilic compounds that contain a positive charge located within or close to a large aromatic ring structure (2042). Plant toxins such as nicotine and quinidine are examples of type II OCs.

Much of what is known of type I OC transport in insects has been the result of studies of secretion of TEA by the Malpighian tubules. Uptake of TEA across the basolateral membrane has been inferred from microelectrode measurement of basolateral membrane potential and the uptake of ^{14}C-labelled TEA (1533). Uptake is electrogenic; depolarization of V_{bl} through increased K^+ in the bathing saline reduces uptake, whereas hyperpolarization of V_{bl} with lower K^+ in the bathing saline enhances uptake. Addition of TEA to the bathing saline depolarizes V_{bl}, consistent with uptake through a conductive pathway. TEA is not taken up through K^+ channels because blockade of K^+ channels with Ba^{2+} does not alter the effect of TEA addition on V_{bl}.

TEA-selective microelectrodes based on the cation exchanger tetra(p-chlorophenyl) borate have been used to measure transport of TEA by isolated Malpighian tubules and guts (Fig. 11). This exchanger was originally used for K^+ measurement, but its selectivity for TEA exceeds that for K^+ by a factor of 10 million. The concentration of TEA in nanoliter droplets of fluid secreted by Malpighian tubules set up in the Ramsay assay can be measured by placing TEA-selective and reference microelectrodes in the droplets. TEA secretion rate (pmol min^{-1}) is then calculated as the product of fluid secretion rate (nl min^{-1}) and secreted fluid TEA concentration (mmol l^{-1}). It is worth noting that the contribution of transporters to elimination of toxins by the Malpighian tubules cannot be unequivocally determined using only measurements of lumen to bath ratios of toxin concentration. Changes in the rates of fluid secretion rate influence the concentration of the solute in the lumen, particularly if the permeability of the tubule wall to the solute is high. A stimulant of fluid secretion will thus appear to inhibit transport of a substrate if only lumen-to-bath concentration ratios are measured, since the concentration of the solute in the lumen will be reduced. In fact, the transepithelial flux of the solute may actually increase, since the reduction in lumenal concentration will reduce the extent of passive diffusional backflux of the solute from lumen to bath. For this reason it is essential to measure both the concentration of the transported solute in the lumen and the fluid secretion rate, so that the transepithelial flux may be calculated.

This reservation applies for measurement of toxin secretion using TEA-selective or salicylate-selective microelectrodes (described below), and also when OC or OA transport is measured by the concentrations of fluorescent transport substrates using fluorescence or confocal microscopy. Some transport substrates are associated with increases or decreases in fluid secretion rate. Rhodamine 123, for example, inhibits fluid secretion rate at concentrations well below the concentration associated with half the maximal transport rate (K_t). Thus, although the luminal concentration is high, the rate of transport is quite low. Measurement of luminal fluorochrome concentrations is complicated by quenching, reflection or absorption of the exciting and/or emitted light by the tissues and by opaque uric acid crystals present in the lumen. It is not possible to clearly visualize fluorochromes and the lumen of Malpighian tubules of *Drosophila* tubules because of opaque concretions in the cells and/or lumen (1023).

Further complications arise when the concretions move in the lumen during fluid secretion, thereby altering the amount of laser light passing through the lumen, or when concretions may be transferred from the cell to the lumen, particularly when fluid secretion is stimulated. Collection of secreted fluid droplets after concretions have settled allows a clean sample of secreted fluid to be collected, and fluorochrome concentration can be measured by taking a sample of the secreted fluid up in a rectangular glass capillary that is optically flat, allowing the fluorochrome concentration to be readily measured by confocal laser scanning microscopy.

TEA-selective microelectrodes can also be used in conjunction with the scanning ion-selective electrode technique to measure TEA fluxes across specific regions of the Malpighian tubules, the ureter that connects the tubules to the gut in some species, and across the gut itself. In *Drosophila*, TEA is transported at high rates by the proximal segment that also reabsorbs KCl and water and at lower rates by the fluid secreting main segment, but not by the distal, non-fluid-secreting distal segment that sequesters Ca^{2+} as luminal concretions. In the Ramsay assay, the concentration of TEA in the lumen of the main segment is ~12-fold higher than that in the bathing saline. Given that the lumen is at an electrical potential of 30 mV to 80 mV positive to the bath, TEA secretion is a thermodynamically active process. TEA uptake is competitively inhibited by other type I OCs, including other quaternary ammonium compounds, cimetidine and quinine. TEA uptake is also inhibited by the type II OCs verapamil and nicotine, suggesting that the basolateral OC transporter has a very broad substrate specificity.

Alteration of excretory mechanisms in response to dietary exposure to toxins

An increased capacity of the Malpighian tubules to secrete toxins is seen after dietary exposure of insects to the same or related toxins. The adaptive value of such an increase is presumably that the insect does not incur the high metabolic cost of maintaining transporters that are not required in the absence of toxin exposure.

When larval *Drosophila* are exposed to 100 mM TEA in the diet, the concentration in the hemolymph increases to ~ 3 mM. Malpighian tubules isolated from larvae reared on diet containing 100 mM TEA secrete TEA at rates approximately 77% higher than those seen in tubules isolated from larvae reared on a TEA-free diet (132). Clearance of TEA from the hemolymph is slowed if the larvae are exposed simultaneously to TEA and cimetidine, a competitive inhibitor of TEA transport by the tubules and posterior midgut.

Expression of P-glycoproteins (P-gp's) in insects is also influenced by dietary exposure to transporter substrates. Both the gut and the Malpighian tubules are important sites of P-gp expression in insects. In *D. melanogaster*, P-gps are the products of expression of three genes, MDR49, MDR50 and MDR65. Increased MDR expression is evident in all sections of the gut (anterior and posterior midgut, hindgut and Malpighian tubules) when larvae of *D. melanogaster* are fed diets containing a non-toxic level of the P-gp substrate colchicine (10 micromolar), relative to control larvae raised on colchicine-free diet (1807).

By contrast with organic cations, dietary exposure to organic anions results in effects on both rates of OA transport and also the rate of fluid secretion (1589). Because fluid secretion is a passive osmotic consequence of secretion of inorganic ions (Na^+, K^+, Cl^-) into the tubule lumen, these findings indicate that exposure to dietary organic anions leads to increased expression or activation of transporters such as the V-type H^+-ATPase, which energizes transepithelial ion transport. Salicylate influx across the posterior midgut, ileum and rectum is not altered in larvae chronically exposed to dietary salicylate. Thus, absorption by the gut is not downregulated in response to excess dietary salicylate. Instead, excretion is enhanced by upregulation of Malpighian tubule salicylate transport. Measurement of Michaelis-Menten parameters in tubules of larvae reared on salicylate-rich diets suggest that transport rate is increased by an increase in the number of transporters (as seen in the increase in the Michaelis-Menten parameter J_{max} from 5.8 pmol min^{-1} $tubule^{-1}$ in tubules from larvae on the control diet to 28.1 pmol min^{-1} $tubule^{-1}$ in tubules from larvae fed salicylate-rich diets). It appears that these transporters are different than those present in the controls, because transport shows a lower affinity for salicylate ($K_t = 0.42$ mmol l^{-1}) relative to tubules from larvae reared on the control diet ($K_t = 0.09$ mmol l^{-1}) (1589).

Malpighian tubules isolated from larvae reared in salicylate-rich diets larvae are apparently modulated through the insertion of more ion transporters into the cell membranes, rather than through an increase in the basal level of intracellular second messengers (cAMP or Ca^{2+}) for diuretic factors. An increase in the number of V-type proton pumps in the apical membrane of the principal cells will therefore lead to an increase in secretion of both cations (Na^+ and K^+) and Cl^-, with a consequent increase in the rate of fluid secretion. Exposure to dietary toxins may thus result in a remodeling of the epithelium, with more and/or different transporters produced by transcriptional, translational or post-translational events. Such phenotypic plasticity is also seen in response to variations in dietary salt intake for *Drosophila* larvae (1287, 1288) or to alterations in ambient salinity for mosquito larvae (443).

Type I organic anions are particularly effective in producing changes in fluid secretion rate and OA transport rate. Exposure to *D. melanogaster* larvae to salicylate-rich or fluorescein-rich diets for only 24 hours is sufficient to cause a nearly twofold increase in fluid secretion rate and in salicylate transport, but also in the tubule secretion of methotrexate, a type II OA (265). MTX transport is Na^+ independent and has distinct transport kinetics (lower K_t, lower J_{max}) relative to salicylate transport, so different transporters are involved. By contrast, fluid secretion rate and MTX secretion increases after 10 days of exposure to dietary MTX, and there is no associated increase in salicylate secretion. These findings suggest

a preeminent role for type I OAs in signaling the increased basal rate of fluid secretion and OA transport rate (265). Much of the increase in the rate of salicylate or MTX secretion is a response to an increase in fluid secretion rate. Tubules of *Drosophila* have a relatively high permeability to these molecules, and so there is a tendency for passive backflux in response to the high concentrations in the lumen and the lower concentrations in the hemolymph. A twofold increase in fluid secretion rate will thus halve the concentration of the OA in the tubule lumen, and there is a consequent reduction in the gradient driving passive backflux. The net effect is an increase in secretion of the OA by the tubule. A similar response is seen when fluid secretion rate is increased in response to addition of diuretic factors or their second messengers; the reduction in diffusive backflux results in increased net secretion of organic anions (1343). It is important to emphasize that this phenomenon is dependent on a relatively high permeability of the tubule wall to small molecules. Tubules of the blood-feeding hemipteran *Rhodnius* have a much lower permeability to small molecules, so that there is little diffusive backflux, and changes in fluid secretion rate do not alter the rates of passive or active secretion of small molecules by the tubule (1131).

It is worth noting that a very different strategy of dealing with potentially toxic material is seen in the midgut of lepidopteran and mosquito larvae. The plant detritus on which the larvae feed contains tannins, which are potentially toxic because they complex freely with enzymes, thereby disrupting insect digestion by immobilizing gut enzymes via nonspecific blockage of active sites (109). High pH in the lumen of the anterior midgut favors dissociation of tannin-protein complexes and is also optimal for digestive enzyme function in the larvae. An intriguing aspect of midgut physiology of larval mosquitoes is the presence of the regional variations in luminal pH. From near-neutrality in the foregut, luminal pH increases in the anterior midgut to >10, one of the highest recorded values in animals, then declines to 7.5 in the posterior midgut.

Much of our knowledge of the physiological mechanisms of alkaline pH generation in the midgut lumen is derived from the extensive studies by Moffett, Onken and coworkers (1376). Lumenal alkalinization is energized by a V-ATPase in the basal membrane (1407) transferring protons from cell to hemolymph. The neurohormone serotonin stimulates the V-ATPase activity, leading to surprisingly high intracellular pH values (8.58) (1377). However, the means by which acidic equivalents are transferred across the apical membrane (from lumen to cell) are much less clear. Inhibitor studies suggest that carbonic anhydrase is not involved in supplying acid-base equivalents to the respective transporters. Surprisingly, the Na^+/K^+ ATPase is present in the apical (lumen-facing) membrane of the gut but appears not to be involved in driving luminal alkalinisation (1378), which is maintained in the presence of ouabain. Pharmacological studies also rule out the involvement of apical Cl^-/HCO_3^- exchangers, apical H^+ channels and apical cation/proton exchangers (1376).

Transport and excretion of divalent ions and bicarbonate

Calcium Insects regulate the level of Ca^{2+} in their extracellular fluids by a mechanism fundamentally different from that operating in vertebrates. Calcium homeostasis in mammals results from precise control of transcellular calcium absorption by the duodenum. By contrast, control of excretion and not absorption is the major means of hemolymph calcium regulation in flies (*Calliphora vicina*) (1810). In response to Ca-rich diets, the calcium content of *Ca. vicina* rapidly increases to more than double the original content but then remains stable. However, midguts isolated from flies fed either Ca-rich solutions or water absorb calcium at the same rate. It appears that rapid calcium absorption from the blowfly midgut is necessary to remove calcium from the lumen so that other processes such as phosphate absorption can operate. Regulation of hemolymph Ca^{2+} is accomplished not by adjustments of midgut absorption but through increases in the rate of Ca^{2+} removal by the Malpighian tubules. The tubules of dipterans remove calcium from the hemolymph through the processes of secretion and sequestration. Approximately 85% of calcium that enters the tubule is sequestered, and 15% is secreted in soluble form into the tubule lumen. Although adult fruit flies may ingest 30 times more calcium than they retain, hemolymph calcium concentration and whole animal calcium content are precisely regulated. A 620% increase in dietary calcium level, for example, is associated with an increase in whole fly calcium content of only 10%, and hemolymph calcium concentration (~0.5 mM) is similar in flies raised on diets differing more than sixfold in calcium content (454). The main segment of the Malpighian tubule of *D. melanogaster* contributes to this regulation by secreting an amount of Ca^{2+} equivalent to the entire body Ca^{2+} content in approximately 9 hours. Much more rapid elimination of calcium is achieved through sequestration of Ca^{2+} as concretions of bicarbonate and phosphate in the distal segment of the anterior pair of tubules. The two distal segments can sequester an amount of calcium equivalent to the body Ca^{2+} content in approximately 2 hours (454).

Secretion and sequestration of calcium by the tubules are independent processes. Ca^{2+} sequestered within the tubule is released into the lumen even when basolateral uptake is blocked. Whereas cAMP increases both basolateral uptake and transepithelial secretion of Ca^{2+}, the calcium channel blockers diltiazem and verapamil inhibit only basolateral uptake (453). A role for Ca^{2+} uptake through channels is suggested by increases in uptake of Ca^{2+} across the basolateral membrane when basolateral membrane potential is hyperpolarized by bathing tubules in saline with reduced K^+ concentration. Transport from cell to lumen is thermodynamically uphill against opposing chemical and electrical gradients and must involve some form of Ca^{2+} pump, either an ATPase or an electrogenic exchanger (e.g., $3Na^+$:Ca^{2+}). Transepithelial Ca^{2+} secretion is increased by treatments that depolarize the transepithelial potential by increasing Cl^- conductance.

Exposure to bicarbonate-free saline acidifies the secreted fluids and also enhances transepithelial Ca^{2+} secretion, possibly through increased dissolution of intracellular granules through changes in intracellular pH.

By contrast with dipteran tubules, the Malpighian tubules of *Rhodnius* sequester almost all of the Ca^{2+} ingested (1139). Avian blood contains 1.5 mM Ca^{2+}, and the insect ingests a blood meal equivalent to ~10 times its unfed weight, so Ca^{2+} is present in considerable excess. The rationale for sequestration rather than secretion may be to avoid exposure of hindgut transporters to high levels of Ca^{2+}, which might interfere with rectal ion and water transport. Alternatively, calcium deposits in the tubules may subsequently be mobilized for production of eggs or spermatophores after ecdysis of adults. Rates of calcium uptake by the tubules increase several days after the blood meal, coincident with release of Ca^{2+} from the digested red blood cells. The post-prandial increase in tubule Ca^{2+} uptake may be an example of an inducible transport system, stimulated by some correlate of feeding. Similar increases in tubule secretion of organic anions such as para-aminohippuric acid and uric acid are also seen after the blood meal. It is also worth emphasizing that the calcium concretions in the tubules are dynamic structures rather than static precipitates. ^{45}Ca that has been incorporated in the previous 48 hours into the tubules is released at the rate of about 25% per hour when the tubules are transferred to ^{45}Ca-free saline. Sequestration of calcium is thus a continuous and metabolically dependent process. Calcium becomes less accessible to the hemolymph over time, presumably since the "oldest" calcium is near the center of the concretions. Tubules of larval *Rhodnius* and crickets are relatively free of intracellular Ca^{2+} concretions immediately after ecdysis, suggesting that the concretions are eliminated during the moult cycle. Cricket tubules also transfer intracellular calcium concretions into the lumen in response to stimulation with cAMP, a second messenger for CRF-related diuretic hormones (721).

Bicarbonate An extreme example of bicarbonate excretion is seen in the rectal salt gland of the larvae of the mosquito *Aedes dorsalis*. The larvae are found in alkaline (pH 10.5) lakes containing high levels of bicarbonate (250 mM) and carbonate (100 mM). Although filter feeding and replenishment of water lost by osmosis across the integument entail ingestion of the medium at rates of 130% body weight per day, the larvae maintain hemolymph bicarbonate concentration at 8-18.5 mM and hemolymph pH at 7.55-7.70 (1783-1785). It is suggested that the salt gland normally secretes a NaCl-rich fluid into the lumen; the posterior and anterior segments secrete approximately 75% and 25% of the total, respectively. The anterior segment of the rectal salt gland is involved in Cl^- uptake (via Cl^-/HCO_3^- exchange). Bicarbonate and carbonate are excreted into the lumen of the posterior rectum against both chemical and electrical gradients. However, both salt gland segments are capable of secreting strongly hyperosmotic fluids containing high concentrations of Na^+, Cl^-, and HCO_3^--CO_3^{2-} (169-172, 1785). The fluid is then modified by ion exchange and reabsorption processes to suit the osmo- and ion regulatory requirements of the larva.

Toxic metals

Insects are relatively insensitive to cadmium, copper, lead, nickel and zinc in acute laboratory toxicity tests, with LC_{50} values typically about four orders of magnitude higher than concentrations found in nature (199). Populations of *Chironomus riparius* that have adapted to high levels of cadmium in the water both accumulate more cadmium and show higher rates of cadmium elimination when transferred to cadmium-free water (1472). Cadmium is first sequestered by metal-binding proteins, then stored in the form of membrane-bound concretions in the posterior midgut of *Chironomus* and subsequently expelled into the lumen by exocytosis or degeneration of whole cells (352, 1645). The gut does not act as a complete barrier, allowing access of Cd to the hemolymph (1033). The anterior midgut is the main site of Cd entry into the hemolymph, whereas the posterior midgut absorbs Cd from the gut lumen as well as from the hemolymph. Cadmium is also both secreted by and sequestered within the Malpighian tubules. Although sequestration is the major mechanism at high levels, secretion is the dominant form of detoxification by the Malpighian tubules at levels closer to those that are environmentally relevant (10 μmol l^{-1} or less). Experiments with isolated tissues suggest that movement of Cd into the hemolymph across the anterior midgut is balanced by movement of Cd toward the lumen by the remaining gut segments and by secretion and sequestration by the Malpighian tubules. As a consequence, levels of Cd do not tend to increase further.

Although high doses of Zn and Cu are toxic, both metals are components of many enzymes, and their levels in tissues of the fruit fly *Drosophila* represent regulated storage rather than deposit excretion. Zinc is accumulated primarily in the main segments of both the anterior and posterior Malpighian tubules at concentrations up to 2.8% of tissue dry weight (1637).

Limiting Respiratory and Cuticular Water Loss

The success of insects in the terrestrial environment is something of a paradox given that their small size and consequently large ratio of surface area to volume should render them vulnerable to desiccation. An appreciation of the desiccating stresses imposed on small terrestrial animals such as insects can be gained by comparing exposure to subsaturated air with exposure to the osmotic pressures experienced in hypersaline aquatic environments. The osmotic pressure (OP) in mosmol/kg equivalent to a specific % relative humidity (%RH) is given by the equation:

$$OP = RT/V(\ln(\%RH/100)) = 55(\ln(\%RH/100)) \times 10^3$$

Vertebrate body fluids (~300 mosmol/kg) are thus in equilibrium with 99.5% RH, and seawater is in equilibrium with

98.2%RH. The Dead Sea (~33.7% salinity; ~ 10 osmol/kg) provides an osmotic pressure approximating 90% RH.

Given these extraordinarily desiccating conditions in the terrestrial environment, there has been strong selection pressure for limiting water loss during the evolution of insect life. Water can be lost in multiple ways: through the respiratory system by evaporation through open spiracles; through water associated with elimination of urine or feces or present in eggs or cocoons; or by transpiration across the cuticle of the exoskeleton.

Cuticular water loss

Insects provide examples of some of the most impermeable body surfaces known, and desert species such as tenebrionid beetles minimize transpiration in spite of exposure to high ambient temperature and low relative humidity. Although respiratory water loss is significant in active insects, more than 80% of water is typically lost across the cuticle (607). The primary barrier to evaporative water loss through the cuticle is a thin (< 1 um) layer of epicuticular lipids. The lipids consist primarily of hydrocarbons in most species, but wax esters, ketones, alcohols and sterols have also been identified. The importance of lipids in providing a barrier to water loss is evident in the 10-100-fold increase in water loss rate after surface lipids are removed by brief exposure to organic solvents (683). Although solvents may also alter respiratory water loss, cuticular transpiration can be isolated by measuring water loss when CO_2 release is negligible (i.e., when the spiracles are closed). The phase transition model proposes that cuticular lipids exist in a solid state at moderate temperatures and can thereby form an effective barrier to cuticular transpiration (606). However, the lipids melt and become more permeable as temperature increases; as a consequence, water loss also increases rapidly. The n-alkanes in insect cuticle typically have chain lengths of 20-40 carbons. Insertion of *cis* double bonds or addition of one or more methyl groups will alter both the melting point and water permeability. In addition, some cuticular lipids are more polar and contain alcohols, aldehydes, ketones and wax esters. In general, one might expect increases in hydrocarbon chain length within or between species to be associated with decreases in permeability, on the assumption that water molecules must diffuse across the hydrophobic interior of a lipid layer during transporation. *A priori*, one might also expect that increasing the degree of unsaturation would decrease the effectiveness of molecular packing and thus lead to an increase in water permeability. However, experimental evidence shows that chain length is the least important factor affecting the melting point of cuticular lipids; methyl branching or insertion of ester linkages or *cis* double bonds tends to have far more dramatic consequences. The effects of methyl-branching depend on the position of the branch point; shifting the branch from a terminal to an internal position decreases T_m (604, 606).

Most insects have complex mixtures of cuticular lipids; unfortunately, we know relatively little about how different compounds interact to determine cuticular permeability. However, Fourier transform infrared spectroscopy (FTIR) can be used to analyze the temperature dependency of lipid phase behavior, and such studies allow inferences to be drawn about the effects on water permeability. Studies of two component mixtures of hydrocarbons by FTIR do not reveal biphasic melting transitions expected on the basis of independent phase behavior of the component lipids. Instead, melting occurs over a broader temperature range than pure hydrocarbons (603) and is associated with increased cuticular water permeability (605, 1581).

Because transpiration increases dramatically above a species-specific "transition temperature" in some insects, it has been suggested that there might be a corresponding phase change in the cuticular lipids. An abrupt change in slope of a plot of water loss rate as a function of temperature was interpreted as an indication of a temperature-induced alteration of cuticle structure. The point where the slope changes is referred to as the critical transition temperature (CTT) in Arrhenius plots of the logarithm of water loss rate (WLR) against the reciprocal of temperature (T):

$$\ln \mathrm{WLR} = -E_a/RT + \ln A,$$

The slope ($-E_a/R$) is the activation energy (E_a) divided by the gas constant (R) and A is the intercept. In the context of water loss from an insect, E_a is an indicator of the amount of energy that a water molecule needs to pass through the cuticle. Plotting the logarithm of WLR is essential, since apparent CTTs in untransformed data may disappear when the data are displayed in an Arrhenius plot (2054). More importantly, the removal of lipids from the cuticle using solvents or physical abrasion may shift the Arrhenius plots upward on the ordinate but does not change the CTT or E_a. The upward shift on the ordinate is consistent with enhanced water diffusion across the cuticle when the lipids are removed. These findings show that the E_a for water loss on the Arrhenius plots is not a true activation energy, and that the CTT appears unrelated to lipid structure. It is still unclear as to whether the transition temperature for permeability is related to some form of lipid phase transition, or whether partial melting is sufficient to allow increased permeability (603). Early literature also suggested that a specific physical arrangement of lipid molecules in the cuticle, such as an orientated monolayer, might contribute to the reduced water permeability. However, use of a magnetic resonance technique suggests that lipids are unlikely to interact with the cuticle so as to form a monolayer, since the epicuticular lipids do not show a preferred orientation (1842).

In summary, surface lipids are important in determining cuticular transpiration, but other factors have a role to play. The cuticle is a complex structure, and aspects such as the degree of melanization may also alter permeability. Melanin granules are polymers of dopa and other tyrosine derivatives that darken the cuticle. However, melanin may also reduce the permeability of the cuticle because it is hydrophobic.

Respiratory water loss

The respiratory system of insects consists of a ramifying system of tubes (trachea) terminating in tracheoles that may extend into cells and lie against the mitochondria. The tracheal system communicates with the atmosphere through valve-like structures called spiracles. In many insects and other arthropods, the pattern of exchange of respiratory gases between the tracheal system and the atmosphere is discontinuous. These discontinuous gas cycles (DGCs) are based on a pattern of opening and closing of the spiracles.

DGCs generally comprise three phases: closed (C), flutter (F) and open (O). When the spiracles are tightly closed, oxygen consumption by the tissues reduces the partial pressure of oxygen (pO_2) within the tracheae. The buffering capacity of the hemolymph and the 24-fold to 30-fold higher solubility of CO_2 relative to O_2 means that there is not a corresponding increase in gas pressure exerted by CO_2. As a result, pressure within the tracheal system drops below that in the atmosphere. When pO_2 declines to a level of ~2-4 kPa, the flutter phase begins. The spiracles transiently open partially, then close in quick succession; water loss is thought to be limited during F phase because the water vapor that is tending to diffusing outward is entrained in the bulk inward movement of air produced in response to the negative pressures within the tracheal system. Carbon dioxide continues to accumulate within the tracheal system throughout the F phase. When endotracheal pCO_2 reaches a level of ~3-6 kPa, the spiracles open widely, essentially allowing the accumulated CO_2 to be flushed out as the tracheal gases to equilibrate with the atmosphere. Ventilatory movements may aid diffusive gas exchange during the O phase.

Several hypotheses have been advanced to explain the adaptive value of DGCs (1182). The three best known are: (1) the hygric hypothesis, which suggests that DGCs reduce respiratory water loss; (2) the chthonic hypothesis, which suggests that DGCs facilitate gas exchange during environmental hypoxia, hypercapnia or both; and (3) the oxidative-damage hypothesis, which suggests that DGCs minimize oxidative tissue damage.

Each of the models makes specific predictions regarding the lengths of DGCs. The respiratory surfaces are saturated with water vapor, so rates of water loss will be proportional to the saturation deficit, which is influenced by the temperature and water vapor pressure of ambient air. Because saturation deficit is highest at high temperatures and low ambient water vapor pressures, the hygric hypothesis predicts a positive relationship between DGC duration and ambient temperature, and a negative correlation between DGC duration and precipitation. The chthonic hypothesis predicts longer DGC duration in hypoxic habitats. For the oxidative-damage hypothesis, low ambient pO_2 will result in low endotracheal pO_2 values, thus reducing the need for a prolonged C phase to reduce endotracheal pO_2, and a consequent increase in DGC duration. The oxidative damage hypothesis thus predicts a positive association between pO_2 and DGC duration.

A meta-analysis based on published data for 40 wild-caught species revealed a significant positive relationship between DGC duration and habitat temperature and an important interaction between habitat temperature and precipitation. Based on these results, it appears that the hygric hypothesis is supported and that DGCs of insects reduce respiratory water loss but allow for adequate gas exchange (1978). It has also been proposed that DGCs reflect circadian, developmental or artificially induced reductions in brain activity. DGCs may result when the thoracic and abdominal ganglia regulate ventilation in the absence of control from higher neural centers, possibly corresponding to a sleeplike state in the insect (1182).

Water Vapor Absorption

The small size of insects and the resulting large ratio of surface area to volume mean that they are in danger of desiccation in the terrestrial environment. Water is lost by transpiration across the body surface, through respiration and through elimination of wastes by the gut. Given the potential for water loss on land, it is perhaps counterintuitive that insects have become the dominant terrestrial group of animals in terms of numbers of species and individuals. In addition to sophisticated mechanisms for reducing cuticular and respiratory water loss, some insects are able to gain water by condensing atmospheric water vapor. Flying insects can readily and quickly access water sources separated by distances that are large relative to the insect's dimensions. For many non-flying species or larvae, however, the distances between liquid water sources may be prohibitive; water sources only a few tens of meters distant may be unobtainable for a slow-moving species that is only a few millimeters in length. But although sources of liquid water may thus be unobtainable, the high rates of gaseous diffusion mean that the insect is continually exposed to water in the form of vapor. The importance of water vapor absorption as a terrestrial adaptation is evident in the independent evolution of this phenomenon at least nine times. Different sites and fundamentally different mechanisms are involved, as seen in the examples listed in Table 5.

The repeated evolution of WVA mechanisms may be related, in part, to the relatively low metabolic cost of the process, even in the face of thermodynamic gradients that are very large. The gradients can be appreciated by expressing the minimum % relative humidity (%RH) at which water vapor is absorbed with an equivalent osmotic concentration (C_{osmol}, osmol/kg) using the equation given above in the first paragraph of the section "Limiting Respiratory and Cuticular Water Loss." For an insect such as the desert cockroach, absorbing water vapor at 82.5%RH, the equivalent osmotic concentration is 11.8 osmol/kg, approximately 10 times that produced by electrolyte concentration in the loop of Henle in the human kidney. For species such as the firebrat *Thermobia*, absorbing water vapor at 43%RH, the osmotic gradient is > 70 osmol/kg.

Table 5 Sites and Mechanisms of Water Vapor Absorption in Terrestrial Arthropods

Species	Site	Proposed Mechanism	References
Desert cockroach (*Arenivaga investigata*)	Mouthparts (Hypopharynx)	Hydrophilic cuticle	(1330, 1335, 1336, 1339)
Ticks (*Ixodes ricinus*)	Mouthparts (Hypostome)	Hydrophilic cuticle	(572)
Booklice (Psocoptera) (*Ctenolepisma sp.*)	Mouthparts	CLVP: Labial glands	(1585)
House dust mites	Coxal glands	CLVP: Hyperosmotic secretion (NaCl, KCl)	(50)
Millipede (*Polyxenus lagurus*)	Cryptonephridial complex	CLVP: Hyperosmotic secretion	(2038)
Mealworm (*Tenebrio molitor*)	Cryptonephridial complex	CLVP: Hyperosmotic KCl	(1122, 1332, 1505)
Flea (*Xenopsylla cheopis*)	Rectum	Unknown	(114)
Thysanurans (*Thermobia domestica*)	Evaginated rectal sacs	Electro-osmosis	(984, 1324)
Isopods (*Porcellio scaber*)	Abdominal pleopods	CLVP: Hyperosmotic NaCl	(2037)
Collembolans	Body surface	CLVP: Sugars and polyols	(793)

Abbreviation: Colligative lowering of vapor pressure (CLVP)

In spite of the large gradients involved, the metabolic cost of WVA is comparatively low; absorption of 6% body weight per day by the desert cockroach requires an energy expenditure equivalent to less than 1.5% of the insect's basal metabolic rate (474). Similarly, absorption by *Thermobia* for 1 hour requires metabolism of as little as 18 μg of glucose, or less than 0.07% of the body weight. For comparison, flying insects may consume fuel at a rate of 0.6% to 20% of the body weight per hour (1128).

Mechanisms of WVA proposed for most species rely on colligative lowering of vapor pressure at a specific site through production of highly concentrated organic or inorganic solutes. In these schemes, energy is required for concentrative solute transport, atmospheric water vapor condenses and the solutes are recycled after the condensed water is transferred into the hemolymph.

The rectal complex of tenebrionid beetle larvae provides the best characterized example of solute-coupled WVA. The species that have been studied include the common mealworm larvae of *Tenebrio molitor* as well as the larvae of several Namibian desert species in genus *Onymacris*. The adults of the Namibian beetles derive water from coastal fogs. Adult *Onymacris unguicularis*, for example, collect dew on their dorsal body surface and conduct it to the mouth; other species collect fog water droplets that have condensed on vegetation, or collect the water that condenses on the raised ridges of trenches they have dug in the sand (690).

In contrast to the collection of fog water from saturated or near-saturated humidities by adult *Onymacris*, several species of tenebrionid beetle larvae can absorb water vapor from humidities as low as 84% RH in the case of *Onymacris marginipennis* (1122). The driving force for WVA is the production of a highly concentrated solution of KCl within the lumen of the Malpighian tubules, as determined in the original micropuncture measurements of Ramsay (1505) and subsequent measurements of lumenal $[Na^+]$, $[K^+]$, $[Cl^-]$ and pH using ion-selective microelectrodes (1122, 1332). WVA is accomplished by the rectal complex, which consists of the distal ends of the Malpighian tubules applied to the outer surface of the rectum and enveloped in the perinephric membrane. The multiple layers of flattened cells that form the perinephric membrane provide a water-impermeable barrier between the high solute concentration the Malpighian tubule lumina and the hemolymph. As a consequence, water or water vapor within the rectal lumen is at a higher activity than within the Malpighian tubule lumina. Water flows across the intervening rectal epidermal cells, which act as a passive osmotic coupling, and into the lumen of the tubules. The osmotic gradient established within the rectal complex is involved both in recovery of water from the fecal pellets within the lumen and atmospheric water vapor absorption. Electrochemical gradients calculated from measurements with ion-selective microelectrodes indicate that the proximal source for ions transported into the tubule lumen is the perinephric space. Ions may pass from the hemolymph to the perinephric space through the thinner and more permeable anterior perinephric membrane (1333).

Most WVA mechanisms depend on active epithelial ion transport to produce concentrated solutions with the required reduction of water vapor pressure. In tenebrionid beetle larvae, for example, condensation of atmospheric water vapor in the rectum is a passive and secondary consequence of primary active salt secretion into the lumen of the Malpighian tubules of the rectal complex. Energy is thus required to

drive ions thermodynamically uphill from lower concentrations in the hemolymph to higher concentrations in the tubule lumen.

In the desert cockroach, however, WVA appears to be a passive consequence of the hydrophilic properties of specialized mouthparts, and energy is required to effect the release of water after condensation. Water vapor is absorbed at humidities above 82.5% RH. An adult female can absorb 20 mg day^{-1} at 96% RH, equivalent to about 4% of body mass per day. During vapor uptake, atmospheric water vapor condenses onto two bladder-like diverticula of the hypopharynx that protrude from the mouth. The uptake is measured as weight gain when an animal is placed in a continuously recording electronic microbalance under conditions of controlled temperature and humidity. Surface temperatures of the bladders, measured by miniature thermocouples, are warmer than the surrounding mouthparts. This temperature difference is produced by heat release during the vapor-liquid phase change during WVA and is humidity dependent (1339). Greater condensation in higher humidity results in increased rates of weight gain and higher bladder temperatures.

A pair of non-epithelial structures situated beneath the frons in the head produces a fluid applied to the bladders during WVA. This fluid is an approximately isoosmotic ultrafiltrate of the hemolymph and does not provide an "osmotic sink" to drive atmospheric WVA. The frontal bodies that produce the fluid are thickened derivatives of the integument. Cyclical contractions of an enveloping muscle mass distort the frontal bodies and produce internal pressure changes sufficient to extrude fluid through a porous cuticular plate at the oral end of each frontal body. A groove in the epipharynx connects each frontal body to the antero-dorsal region of the bladder surface (1335).

Analyses of the frontal bodies and the fluid on the bladder surface indicate that the total concentration of salts on the bladder surface is less than 275 mEq, and the sum of the concentrations of organic solutes (sugars, amino acids, polyhydroxyl alcohols) is less than 2 mM. The fluid produced by the frontal bodies is thus not hygroscopic and it must play a subordinate role in the WVA mechanism of *Arenivaga* (1330, 1336).

A speculative model for the mechanism of WVA in the desert cockroach proposes that reduction of water activity on the bladder surface is due to the properties of the fine cuticular hairs, 0.1 mm in length and as fine as 180 nm in diameter, which cover the bladders. Isolated samples of bladder cuticle were shown to be hydrophilic by measurement of their water content over a range of humidities (1336). In other words, the water content of bladder cuticle (g H_2O/g dry weight) is much higher than that of unspecialized cuticle from other regions of the body. Importantly, the water content of the hairs, measured gravimetrically or inferred from changes in the volume of the hairs measured by scanning electron microscopy, is highly sensitive to small changes in ionic strength. Polyelectrolytes, such as chitin and protein in the bladder cuticle, swell as the ionic strength of the surrounding medium is reduced (1804). Increases in salt concentration reduce the mutual repulsive forces on a protein or gel structure and permit a greater number of non-covalent cross-links, possibly of the van der Waals type, to form. As a consequence, the protein or gel shrinks (888). Cyclical addition of frontal body fluid may sufficiently alter ionic strength to promote shrinkage of the bladder hairs, thereby releasing water from the hairs. Some of this released water may evaporate, but much will be swallowed by the substantial negative pressures generated by the animal to pull fluid and condensate over the bladder surface and into the esophagus. After the pulse or wave of increased ionic strength passes over a particular area of the bladder surface, the hydrophilic cuticular hairs will tend to swell again by absorption of atmospheric water vapor. In other words, condensation will dilute the surrounding fluid, leading to further swelling, until swelling is constrained by the elastic properties of the cuticular hairs (1333).

Soil-dwelling collembolans also provide an example of a very different basis for water vapor absorption. Collembolans are hexapods with internal mouthparts and are now considered closely related to, but distinct from, the insects. Respiratory gases are exchanged across the integument, whose permeability to water is high. They are thus in constant danger of dessication and survive only hours at relative humidities below 90%. However, they live in the pores between soil particles where the humidity is typically very close to 100% RH. Accumulation of substantial quantities of sugars such as glucose, trehalose and polyols such as myo-inositol raises the osmotic pressure of their body fluids. The consequent reduction in water vapor pressure allows passive absorption of water vapor from the surrounding atmosphere at humidities above 95%, the lethal limit for drought tolerance in these animals. In contrast to the insects, which are characterized by cuticle with very low permeability to minimize water loss and in which the mechanisms of water vapor absorption entail localized creation of extremely low water activity in specialized tissues, collembolans have a highly permeable integument, making localized active water absorption inappropriate. As a result, these animals must maintain all of the body fluids hyperosmotic to the surroundings to allow net water uptake from the atmosphere by passive diffusion along the gradient in water potential (793).

Agnatha and Pisces

All three possible strategies for maintaining salt and water balance are represented by fish in marine environments. (i) Osmoconformity/ionoconformity is found in the marine agnathan hagfishes, which do not regulate osmotic pressure and concentrations of main electrolytes to a great extent (1251, 1604). (ii) Osmoconformity but regulation of main ions is seen in marine and some euryhaline elasmobranch (722) and in the lobe-finned coelacanth (649), which, in marine environments maintains plasma osmolality slightly above that of seawater but NaCl concentrations at 30-35% of ambient levels. (iii) The most commonly observed

strategy is osmoregulation, found in all teleosts (1167) and lamprey (1251), which regulates main extracellular ions (Na^+ and Cl^-) and osmotic pressure at approximately 150 mM and 300 mOsm, respectively, regardless of ambient ion concentrations. Hagfish and lobe-finned fish are restricted to marine environments. In freshwater, all fish regulate osmotic pressure and ionic concentrations above that of the environment. Examples of osmotic pressure, ionic and organic constituents of fluid compartments in fish are given in Table 6.

For a discussion of the evolutionary history leading to the phylogenetic distribution of the above osmoregulatory strategies, refer to Evans et al. (513).

Table 6 Composition of Various Body Fluids from Fish

	Hagfish		Elasmobranch				
Environment	Marine		Freshwater	Marine			
Fluid	Plasma[a]	Urine[b]	Plasma[c]	Plasma[d]	Intestinal fluid -posterior[d]	Rectal gland secretion[f]	Urine[b]
Na^+	549	533	178	263	356	540	240
Cl^-	563	548	146	244	369	533	240
Urea	3	9	1	386	22	14.5	100
TMAO	–	–	–	36	6	–	10
Mg^{2+}	19	15	–	1	30	<1	40
SO_4^{2-}	–	7	–	3	13	–	70
Ca^{2+}	5	4	–	3	28	<1	3
K^+	11	11	–	5	8	7	2
HCO_3^-	–	–	–	4	1	–	
pH	–	7.6	–	7.6	6.4	–	5.8
mOsm	–	–	320	947	950	1,018	

	Teleost					
Environment	Freshwater			Marine		
Fluid	Plasma[g]	Intestinal fluid[f]	Bladder urine[g]	Plasma[e]	Intestinal fluid posterior[e]	Bladder urine[e]
Na^+	139	130	4	166	28	34
Cl^-	117	99	6	153	30	26
Urea		–	–	–	–	7
TMAO		–	–	–	–	–
Mg^{2+}		1	1	1	153	95
SO_4^{2-}		1	–	–	130	74
Ca^{2+}	3	2	<1	2	5	–
K^+	4	9	6	4	5	–
HCO_3^-		20	–	5	53	–
pH		–	–	7.8	8.2	–
mOsm	290	325	26	329	329	305

Concentrations of urea, TMAO and electrolytes (mmol/kg or mmol/l) as well as pH and osmotic pressure in various body fluids from fish.
"–"; not determined or reported, [a]; Atlantic hagfish (365), [b]; Pacific hagfish and Spiny dogfish (749), [c]; *Potamotrygon sp.* (2029), [d]; Little skate (34), [e]; Gulf toadfish (1203), [f]; freshwater-acclimated tilapia (653), [g]; Northern pike (749), [f]; Spiny dogfish (224).

Agnata

Hagfish are found exclusively in the marine environment, and their strategy for maintaining salt and water balance have been discussed extensively recently (365, 509). Although hagfish presumably do not need to drink at high rates to maintain water balance, evidence for components of the renin-angiotensin system (RAS) has been reported (324). It is possible that the slightly lower osmotic pressure of the plasma compared to seawater (Table 6) does require some seawater ingestion for hagfish to maintain water balance. Although approximately isoosmotic to seawater, plasma K^+, Ca^{2+}, Mg^{2+}, Cl^- and SO_4^{2-} concentrations are slightly below those of seawater, while Na^+ concentrations seems to be slightly above (Table 6). The transepithelial potential (TEP) across the hagfish integument remains to be reported, but regardless of what it is, some of the aforementioned concentration differences must reflect ions out of electrochemical equilibrium. While extracellular fluids are similar to seawater, although not identical (see below), intracellular fluids contain lower concentrations of especially Na^+ and Cl^- with amino acids (20-70 mmol/Kg) and trimethylamineoxide (TMAO) (~210-230 mmol/Kg), but not urea, contributing to intracellular osmotic pressure (86).

Gills

Although the hagfish gill morphology differs substantially from that found in lamprey, elasmobranchs and teleost, (509) the gill is still arranged to have counter-current blood and water flow for efficient gas exchange and possesses numerous mitochondria-rich (MR) cells. In addition to large amounts of mitochondria, the cells are characterized by extensive basolateral membrane foldings that are continuous with an intracellular tubular system as seen in other fishes. However, deep apical crypts, as seen in marine teleosts, are lacking (509). In agreement with the limited need for branchial Na^+ and Cl^- transport, Na^+/K^+-ATPase expression and enzymatic activity levels are lower than commonly found in marine teleosts (288, 1146, 1862). Despite their limited branchial transport of Na^+ and Cl^-, hagfish are efficient regulators of acid-base balance (476, 1861) via Na^+/H^+ and Cl^-/HCO_3^- exchange systems, suggesting that these systems originaly evovled for the purpose of acid-base balance regulation rather than osmoregulation (505). In agreement with these observations, carbonic anhydrase, Na^+/H^+ exchangers (NHEs) and the vacuolar H^+-ATPase have been localized to hagfish branchial MR cells (288, 291, 476, 1862), which likely serve acid-base balance rather than osmoregulation.

Gastrointestinal tract

Recent studies have revealed modest drinking rates by hagfish held in normal seawater and a stimulated drinking rate following abrupt transfer to 140% seawater (1812). Furthermore, increased concentrations of a non-absorbable marker, polyethylene glycol, MW 4000 (PEG-4000), in intestinal fluids compared to seawater and a reduction in [NaCl] demonstrate the ability to perform solute coupled fluid absorption across the intestinal tract (1812). However, solute coupled fluid absorption by hagfish does not appear to be associated with anion exchange and intestinal HCO_3^- secretion, as is the case for elasmobranchs and teleosts (below) (1812).

Kidneys

The hagfish kidney has 30-40 glomeruli that drain into paired ducts, structurally similar to proximal tubules of other vertebrates (reviewed by 509). However, glomerular pressures are so low that filtration may not occur (524, 1548), suggesting that urine may be formed mainly by secretion (1553). Urine-to-plasma ratios for Na^+, Cl^- and inulin are approximately 1, indicating no water absorption and no secretion of monovalent ions by the renal tubules of hagfish (1265). In contrast, Mg^{2+} and especially SO_4^{2-} are concentrated in the urine (Table 6) (1265), and secretion of these ions may thus drive fluid secretion. A recent study reported that although hagfish are incapable of regulating plasma osmotic pressure, divalent ions appears to be potently regulated (1604), presumably by renal excretion.

Elasmobranchs and Coelacanths

Little is known about osmoregulation by coelacanths, but their blood is approximately isoosmotic with seawater, with ionic composition very similar to the blood of elasmobranchs (649). Coelacanths have what appears to be a salt secretion gland, the postanal gland, which is similar to the rectal gland of elasmobranchs at the structural and ultrastructural levels (1032). Furthermore, the postanal gland of coelacanths, as the rectal gland of elasmobranchs, has high levels of Na^+/K^+-ATPase activity (648) and is likely involved in secretion of Na^+ and Cl^-. Given these similarities, the following discussion of salt and water balance in elasmobranchs likely applies to coelacanths as well.

Marine elasmobranchs maintain plasma osmolality slightly (~20 mOsm) above ambient (790, 1714) and thus gain osmotically "free" water and show very low, although measurable, drinking rates (34, 40, 401). Plasma Na^+ and Cl^- concentrations of marine elasmobranchs are maintained much below those of the environment but higher than in teleosts, with the main osmolyte being urea at concentrations between 300 and 400 mOsm (Table 6). Urea appears to be produced in the liver as well as the skeletal muscle (35, 876).

The denaturing effects of high osmotic pressure and high urea concentrations are offset by high concentrations of methylamines, especially trimethylamine oxide (TMAO). Although subject to some controversy, the capacity for TMAO production appears to be low and restricted to the liver, and high TMAO levels are likely due mainly to effective retention (1856). Glomerular filtration rates and urine flow rates of marine elasmobranchs are relatively high compared to marine

teleosts (512), and the urine is hypoosmotic, with Na^+ and Cl^- being the main osmolytes. The kidney plays a central role in osmoregulation by reabsorbing mainly urea and TMAO while the rectal gland secretes the excess Na^+ and Cl^- load resulting from the inward-directed gradient of these ions. Little salt excretion occurs at the gill, but the branchial epithelium is nevertheless involved in osmoregulation by contributing to urea retention despite substantial urea gradients between the blood and water (see below).

Euryhaline and even freshwater elasmobranchs survive in low salinity waters. Among euryhaline elasmobranchs, the bull shark and the Atlantic stingray maintain elevated plasma levels of urea even when in freshwater, although urea, Na^+ and Cl^- levels are reduced resulting in lower osmotic pressures compared to those observed in seawater-acclimated animals (1452, 1458, 1459). Salt absorption by these two species, when in low salinities, likely occurs via two distinct populations of branchial MR cells. Uptake of Cl^- appears to take place via cells expressing the apical SLC26a4 anion exchanger (Pendrin) and to be driven by basolateral V-type proton ATPases, while Na^+ uptake likely occurs via apical NHE3 and is driven by basolateral Na^+/K^+-ATPase (1454, 1518).

At least two species of elasmobranchs, the Asian freshwater stingray and the stingray from Rio Negro, are capable of osmoregulation in freshwater. The Asian freshwater stingray displays greatly reduced plasma urea levels (44 mM) when in freshwater but maintains a fully functional ornithine-urea cycle and is capable of increasing circulating levels of urea in response to elevated ambient salinity (1803). In contrast, the South American stingray features an inability to retain urea when challenged with elevated salinity and a degenerated rectal gland incapable of salt secretion (600, 1825, 1826, 1828). Although not conclusive, pharmacological evidence suggests that at least Na^+ uptake in this species occurs via ion exchange mechanisms (2029). For a review of osmoregulation by euryhaline elasmobranchs, see Hazon et al. (722).

Gills

The elasmobranch gill epithelium consists largely of pavement cells, although relatively large MR cells are present in interlamellar regions and on the lamellae (289, 1858). Elasmobranch MR cells display microvilli on the apical membrane, basolateral membrane infoldings, a tubulovesicular system in the apical region and express Na^+/K^+-ATPase (509, 2005). Despite the expression of Na^+/K^+-ATPase, elasmobranch gill MR cells are likely not involved in NaCl secretion as evidence for NKCC1, CFTR and K^+ channels, which are all present in the salt-secreting rectal gland (below), have yet to be reported. Although not involved in NaCl secretion, MR cells of elasmobranch gills play a vital role in regulating acid-base balance (1858, 1861, 1863) and contribute significantly to osmoregulation by regulating urea transport. Very low apparent urea permeability of the gill is crucial for maintaining the high plasma-to-water urea gradient (752, 1205, 1406) and is the product of unique phospholipid bilayer composition as well as specific "back transport" of urea from branchial epithelial cells to the blood (531, 1406). The competitive inhibitor of urea transporters, phloretin, increases urea efflux, presumably by blocking a basolateral, Na^+-dependent urea transporter. Transport by the basolateral Na^+-dependent urea transporter is stimulated by ATP and is sensitive to ouabain, and thus relies on Na^+ gradients established by the Na^+/K^+-ATPase (531).

Gastrointestinal tract

Contrary to common belief, elasmobranchs ingest seawater (401, 1814), with drinking rates influenced by extracellular fluid volume and controlled by the renin-angiotensin system (33, 37-39, 41), as in teleosts (below). Very little is known about ingested seawater processing by the elasmobranch gastrointestinal tract, but they are capable of performing solute-coupled fluid absorption across various segments of the intestine (34, 40, 1048, 1814). Absorption of Na^+ and Cl^- appears to drive water absorption, and substantial intestinal HCO_3^- secretion rates suggest that part of the Cl^- absorption occurs via anion exchange as is the case for marine teleosts. However, nothing is known about the nature of the anion exchange proteins and the possible involvement of Na^+:Cl^- cotransporters as seen for marine teleosts (below). The main osmolyte in marine elasmobranchs, urea, appears to be lost across the intestinal epithelium as evident from luminal concentrations as high as 375 mM in the proximal segments of the intestine (34). Interestingly, the majority of this urea is reabsorbed by the distal intestinal segments as the concentration falls to $<$22 mM. Although the mechanism of this urea reabsorption remains to be elucidated, high expression of a urea transporter and a member of the Rhesus-like ammonia transporters (Rhbg) suggest that these transporters might be involved in reclaiming the lost nitrogen (34). Considering that many elasmobranchs are intermittent feeders, nitrogen limitations have been proposed for this group of vertebrates (2023, 2024, 2028, 2030). Indeed, elasmobranchs examined so far show a remarkable ability to retain nitrogen even after feeding (2028), at a time when teleost fish show high rates of ammonia excretion (1816).

Kidney

Tubule anatomy The complexity of the elasmobranch kidney rivals that of mammals, as reviewed recently (509). The nephrons are glomerular followed by five segments generally termed the neck, the proximal, intermediate and distal segments, ultimately leading to the collecting tubules and the collecting duct. From a functional perspective, it is important to observe that these five segments are arranged in four loops such that tubular fluids travel in opposite directions in five closely adjacent tubular segments from the same nephron, forming a renal counter-current system (165, 988).

Glomerular filtration and urine flow rates Glomerular filtration rates average 4 ml/kg/h in marine elasmobranchs (749) and appear to be regulated by the number of filtering nephrons (910, 1655, 1723), as is the case for marine teleosts (below). Urine flow rates are substantially lower than glomerular filtration rates are, demonstrating 60%-85% tubular fluid absorption (749, 910, 1723), rates closely associated with absorption of urea and TMAO. Although secretion may occur in the proximal tubules (120), filtration and reabsorption are the important renal processes for marine elasmobranch osmoregulation (115), and elasmobranch urine is typically hypoosmotic by 50-250 mOsm (222).

Tubular secretion and reabsorption Secretion by the elasmobranch proximal tubule appears to be similar to the active extrusion of monovalent ions, driving fluid secretion, observed in teleost proximal tubules (below) (115, 1612). In brief, transcellular Cl^- secretion is achieved by a basolateral NKCC1 and an apical CFTR-like channel while Na^+ follows via a paracellular shunt. Na^+/K^+-ATPase provides the gradients for operation of basolateral NKCC1, with K^+ recycling across the basolateral membrane being facilitated by K^+-channels (120, 1612). This NaCl secretion mechanism is identical to those observed in the elasmobranch rectal gland (below) and the marine teleost gill (below and Fig. 18). Both Mg^{2+} and SO_4^{2-} concentrations in the urine are well above what could be accounted for by fluid absorption (Table 6), suggesting tubular secretion as in teleosts, but nothing is known about elasmobranch tubular secretion of these divalent ions.

The most efficient absorption by the elasmobranch nephrons is that of urea and TMAO, with more than 90% of the filtered load being absorbed (543). Although urine Na^+ and Cl^- concentrations are similar to plasma concentrations (Table 6), substantial absorption occurs and likely contributes to fluid absorption. Studies on the isolated third loop of the nephron (late proximal and intermediate segment) demonstrated Cl^- absorption via NKCC2 (559), which was supported by observations of NKCC mRNA in elasmobranch nephrons with apical membrane localization of the gene product (131, 2047). Urea reabsorption has long been recognized to be linked to Na^+ absorption in a fixed ratio over a wide range of urine flow rates (1630), and can, in part, be accounted for by solvent drag as ~70% of the filtered fluids are being reabsorbed. However, since urine urea concentrations are well below those of the plasma, excess urea reabsorption must occur. Based on the above observations, it has been proposed that NaCl absorption from the lumen of loop 3 tubules produces hypertonicity in the surrounding extracellular fluids, which in turn creates an osmotic gradient for fluid movement from the lumen of the adjacent loop 4; the resulting increased concentration of tubular urea favors urea movement from the lumen of the collecting tubules to the surrounding tissues (559). Carrier-mediated urea transport by elasmobranch renal tubules has long been assumed (718, 910, 988, 1626, 1630) but is now conclusively demonstrated. A facilitated urea transporter (ShUT) cloned from the spiny dogfish exhibits robust renal expression and phloretin-sensitive urea transport when expressed in Xenopus oocytes (1712) and has since be reported from other elasmobranchs (820, 840). ShUT is found exclusively in the final segments of the tubule (820), which is in agreement with the proposed role for this segment in urea absorption. More recently, two distinct apical urea transporters displaying non-saturable phloretin sensitive urea transport (ShUT) and saturable, phloretin sensitive Na^+:urea cotransport have been characterized from elasmobranch kidneys (1249). Interestingly, both these transporters support the links between Na^+ and urea absorption, since ShUT relies on urea gradients established by NaCl and water absorption while the Na^+:urea cotransporter relies directly on Na^+ transport.

Rectal gland

The function of the spiny dogfish rectal gland in salt homeostasis was first demonstrated in 1960; this organ secretes isoosmotic fluid comprised almost exclusively of Na^+ and Cl^- (Table 6) (224). The concentrations of these monovalent ions are approximately double those of the plasma, and the gland therefore contributes substantially to Na^+ and Cl^- elimination. Although the spiny dogfish can compensate for rectal gland removal by elevated renal salt clearance, the lack of a rectal gland compromises the ability to clear injected NaCl from the plasma (223). Similar findings have since been reported for other elasmobranchs (269, 719).

The rectal gland drains into the distal intestine and secretions exit via the cloaca. The gland is supplied by simple vasculature and secretions are carried by a single duct, making this organ well suited for perfusion experiments on isolated preparations or *in situ* (1693, 1728, 1729). The ultrastructure of the rectal gland has been reviewed comprehensively (1369).

The molecular pathways mediating Na^+ and Cl^- secretion are well known, were summarized recently (509) and are thought to be identical to those described for Na^+ and Cl^- secretion by the marine teleost gill (below and Fig. 18). Hypervolemia stimulates rectal gland secretion via secretion of cardiac natriuretic peptide (1636, 1728, 1729), which is potentiated by local release of vasoactive intestinal peptide from rectal gland nerves (1692). Cardiac natriuretic peptide acts via the guanylate cyclase, protein kinase pathway (680, 1691), while vasointestinal peptide acts via cyclic AMP (312, 722). In addition, gland secretions appear to be correlated with blood perfusion, which is limited under physiological conditions by catecholamine induced vasoconstriction (1682, 1683). Finally, stimulation of the extracellular calcium-sensing receptor CaR causes gland vasoconstriction. Elevated plasma Na^+ and Cl^- concentrations act to impair CaR activation in the presence of constant Ca^{2+} concentrations, and thus release vasoconstriction for increased blood flow and thereby gland secretion (522, 523).

Freshwater Fish

Overview

The following likely apply to freshwater lamprey, elasmobranchs and teleosts, although studies on teleosts are by far the most abundant. Gas exchange by fish is achieved by a large branchial surface area and intimate contact between the blood and the surrounding medium. However, these properties of the gill that allow for sufficient gas exchange despite low oxygen solubility in water, combined with large osmotic gradients between extracellular fluids and the freshwater environment, result in diffusive water gain. Consequently, freshwater fish produce large volumes of dilute urine (see Table 6 for urine composition). Although the renal tubules in freshwater fish are specialized for electrolyte absorption, renal ion loss is unavoidable, adds to diffusive ion loss across the gills and must be compensated by ion uptake. Ions are gained from the diet and by active uptake across the gill epithelium mainly by specialized MR cells.

Absolute ion uptake rates are variable among freshwater organisms, including fish, but exhibit a strong dependence of size (664), presumably due to size-dependent mass-specific metabolic rate and relative gill surface area; the greater the mass-specific metabolic rate, the greater the relative gill surface area and thus diffusive ion loss to be compensated by active ion uptake. An analysis, including 50 studies of Na^+ uptake by freshwater organisms, revealed that 65% of the overall variation can be attributed to size (LOG uptake = 2.87 − LOG(Mass)·0.274, where uptake rates and mass are in nmol/gram/hour and grams, respectively (664). According to this relationship a standard 1 gram fish displays Na^+ uptake rates of ~750 nmol/gram/hour, while a standard 100 gram fish takes up Na^+ at a rate of ~ 210 nmol/gram/hour to maintain homeostasis. Other factors obviously play a role in dictating Na^+ uptake rates, among which are ambient Na^+ concentrations. Affinity and maximal transport capacity of the Na^+ (and Cl^-) uptake pathways of freshwater fish range from 13 μM to 18,500 μM and from 380 nmol/gram/hour to 19,000 nmol/gram/hour, respectively (150, 200, 507, 553, 1143, 1408, 1481). Both parameters are sensitive to ambient ion concentrations and are adjusted as fish acclimate to different ion concentrations with both the uptake affinity and maximal transport capacity increasing as ambient ion concentrations decrease (150, 200).

Gills

Ion transport proteins are detected in MR cells and pavements cells of freshwater fish gills (1168, 2004), suggesting that both cell types may contribute to ion transport across the gill epithelium. However, because pavement cells lack substantial metabolic potential to drive transport, the less abundant MR cells likely contribute most to ion uptake. Indeed, strong correlations have been observed between the relative MR cell exposed apical surface area and Na^+ uptake rates in several species of freshwater fish (200, 1427), strongly supporting this notion.

The morphology (and function) of fish gill MR cells has been reviewed most recently in 2005 (513). Morphologically, electron-dense β-cells and less dense α-cells have been identified in gills of freshwater teleost fish or freshwater-acclimated euryhaline teleost fish (1463, 1464, 1466, 1467). Based on morphological observations on gills obtained from fish in different environments, α-cells were suggested to conduct Cl^- secretion in seawater and Na^+ uptake in freshwater, while β-cells were proposed to be associated with Ca^{2+} uptake in freshwater (1462, 1465). Similarly, more than one MR cell type is present in the gills of freshwater-acclimated lamprey (76, 77, 1252, 1291, 1413).

Na^+ uptake models No less than three models for Na^+ uptake by freshwater fish have been proposed.

(i) The classic model of Na^+/H^+ (NH_4^+) exchange, first proposed by Krogh (973) and since attributed to apical isoforms (NHE2 and NHE3) of the SLC9A gene family, driven by electrochemical Na^+ gradients established by the basolateral Na^+/K^+-ATPase, have been challenged recently due to apparent thermodynamic constraints on Na^+ uptake via this pathway (55, 1403). Nevertheless, occurrence of branchial NHE2 and NHE3 in a high number of species including agnathans (291, 476), elasmobranchs (291, 477, 1858) and teleosts (263, 300, 771, 2004) has been demonstrated. Furthermore, transfer of euryhaline species to lower salinities results in increased expression of NHE2 (*Fundulus heteroclitus*; [1642]) and NHE3 (*Dasyatis sabina*; [290]), transfer of zebrafish to low Na^+-freshwater causes an increased branchial expression of NHE3b (2049), and hypercapnic acidosis stimulates expression of NHE2 in freshwater acclimated rainbow trout (835). In addition, the amiloride derivate, EIPA, which is assumed to be a specific inhibitor of NHEs also in fish (771), inhibits Na^+ uptake in freshwater-acclimated *Cyprinodon variegatus variegatus* and *Cyprinodon variegatus hubbsi* (200). These observations suggests that electroneutral Na^+/H^+ exchange may occur via NHE members of the SLC9A family despite apparently unfavorable conditions. It is possible that activity of the Na^+/K^+-ATPase in the extensively folded basolateral membrane, which creates a tubular system in close proximity to the apical membrane (1445), establish low Na^+-microenvironments allowing for the apical NHEs to function even in freshwater environments (771). Recent studies on zebrafish and medaka link the apical rhesus protein Rhcg1 to the NHE3b isoform in a way where NH_3 appears to be excreted via Rhcg1 and be trapped by conversion to NH_4^+ with protons provided by NHE3b (980, 1666, 2043). In turn, the removal of protons from the apical boundary layer by conversion of NH_3 to NH_4^+ facilitates the continued operation of NHE3b allowing for Na^+ uptake. While

these studies provide additional support for the Na^+/H^+ exchange model, it should be noted that it remains to be seen if the Rhcg1/NHE3 constellation applies generally to freshwater fish.

(ii) An alternative model for Na^+ uptake was proposed based on findings of the vacuolar H^+-ATPase in the apical region of rainbow trout gills (1058-1060) and was later confirmed for several freshwater species by observations of reduced or abolished Na^+ uptake in the presence of the specific inhibitor bafilomycin ($1\text{-}2\cdot10^{-6}$M) (150, 526, 666), although some species clearly lack an apical H^+-pump (200, 1408). Localization of the H^+-pump in the apical region has also been demonstrated for zebrafish (150) and is assumed to fuel Na^+ uptake via Na^+ channels by hyperpolarization of the apical membrane. Searches for an obvious Na^+ channel candidate involved in freshwater fish Na^+ uptake, the epithelial Na^+ channel (ENaC), have so far been fruitless, and ENaC is not present in the teleost genomes sequenced to date, leading to the conclusion that other Na^+ (or unspecific cation) channels are involved in Na^+ uptake. Regardless of the nature of the Na^+ channel involved, it is sensitive to the amiloride derivate, phenamil, at least in some species (229, 664), and appears to be subject to rapid, non-genomic downregulation by elevated ambient Na^+ concentrations (150) much like ENaC in isolated frog skin (562, 589). Interestingly, acclimation to low ambient Na^+ fails to induce increased expression of the H^+ pump, although acidic environments potently induce H^+ pump expression in zebrafish (ZF). These observations suggest that the Na^+ channel rather than the H^+ pump is limiting for Na^+ uptake and illustrates the dual role of the branchial H^+ pump in osmoregulation and acid-base balance in freshwater fish.

(iii) A third proposed model for Na^+ (and Cl^-) uptake involves absorptive Na^+-$2Cl^-$-K^+ (NKCC2) or Na^+:Cl^- cotransporters (NCC). Regardless of which of these cotransporters are considered, thermodynamic considerations are difficult to reconcile with Na^+ (and Cl^-) uptake in freshwater. Nevertheless, the evidence for their presence and contribution to ion uptake is growing. Early observations of bumetanide-sensitive ion uptake by goldfish (1481) have since been supported by immunohistochemical observations of branchial NKCC isoforms in the apical region for freshwater-acclimated fish (772, 773, 829). More recently, NCC was identified in MR cells from zebrafish and tilapia (818, 819) and localized to the apical membrane of certain MR cells in zebrafish. Furthermore, knockdown experiments with zebrafish embryos demonstrated a role for this transporter in Cl^- uptake (1930) while the use of the dye, Na^+ green, implicated NCC in Na^+ uptake by zebrafish as well (502).

The H^+ pump undoubtedly contributes to Na^+ uptake across the apical membrane in some species, possibly depending on ambient NaCl concentrations, and basolateral Na^+/K^+-ATPase (NKA) clearly is important for transepithelial Na^+ transport by maintaining low cellular Na^+ concentrations and directly extruding Na^+ across the basolateral membrane. The NKA is highly expressed in gill epithelia (771, 1051, 1201, 1417, 1905, 2004) and localizes to the basolateral membrane or the tubular system of the MR cells (373, 374, 1451), but demonstrates differential subunit composition dependent on ambient salinity. The α1a and α1b subunit isoforms in the gills of rainbow trout are expressed reciprocally and have been proposed to play a role in Na^+ uptake in freshwater and Na^+ extrusion in seawater, respectively (1540); the α1a isoform is upregulated in a number of salmonids when acclimating to freshwater (238, 239, 1680, 1835). Three amino acid substitutions in transmembrane segment 5 (TM5), TM8 and TM9 separate the α1a isoform from the α1b (857). Together these three amino acid substitutions promote binding of Na^+ over K^+ from the cytoplasm, reduce the Na^+/ATP ratio and the work performed on a single pump cycle and furthermore, inhibit binding of K^+ such that Na^+/H^+ rather than Na^+/K^+ exchange is promoted (857). These changes in the α1a subunit seem to be highly adaptive for osmoregulation in low ion environments by facilitating Na^+ uptake against very steep gradients (10-20 μM in water versus 150 mM in blood). Interestingly, a number of α1 subunits have also been identified in zebrafish and cichlids and show differential expression depending on ambient water ion concentrations (1046, 1833). Although the functional differences of the α1 subunits in zebrafish remain to be fully determined, these observations suggests that mechanisms similar to those discussed for salmonids above may also be present in cyprinids. Furthermore, FXYD proteins co-localize with NKA, are differentially expressed depending on water chemistry and "seawater readiness" in salmonids, suggesting a regulatory role of FXYD on NKA activity (1831, 1837).

Chloride uptake Salmonids (633, 635), cyprinids (150, 526, 977) and cichlids (271, 526) rely on active uptake of Cl^- across the gill epithelium while certain anguillids (153, 633, 659, 929) and centracids (1841) display no Cl^- uptake from freshwater. As discussed above, Na^+:Cl^- cotransport systems may contribute to Cl^- uptake by certain freshwater species. However, early observations by Krogh provided evidence for Cl^- uptake via Cl^-/HCO_3^- exchange (973, 977), an observation that has since been confirmed. Induction of metabolic alkalosis causes an increase in HCO_3^- excretion, Cl^- uptake and an increase in MR cell surface area in rainbow trout, providing direct evidence for Cl^-/HCO_3^- exchangers and their localization to MR cells in the gill epithelium (634, 1426). Two groups of Cl^-/HCO_3^- exchangers are found in the SLC4 (HCO_3^- transporters) and SLC26 (anion transporters) gene families (18, 1258, 1572). Results of *in situ* hybridization (1788) and immunohistochemistry (2004) experiments implicate SLC4a1 (AE1) in Cl^- uptake, although these

observations should be interpreted with caution because heterologous probes and antibodies were used. More recently, SLC26a4 (Pendrin) was identified in freshwater acclimated stingray gills (1454), and SLC26a3, SLC26a4 and SLC26a6 were all identified in developing zebrafish embryos (81). Furthermore, these transporters are regulated at the transcriptional level depending on ambient Cl^- or HCO_3^-, and gene knockdown experiments (morpholinos) demonstrated reduced Cl^- uptake when transcription of SLC26a3, a4 and a6 was blocked (81). Similar experiments on adult zebrafish confirmed that mRNA expression of SLC26 transporters responds to lowered Cl^- and elevated HCO_3^- concentrations in water and shows apparent MR cell localization, and that induced changes in expression mirror absolute and coupled Cl^- uptake and HCO_3^- excretion rates (1430). While the evidence for SLC26 anion exchangers in freshwater fish Cl^- uptake is strong, it is not entirely clear how electroneutral anion exchange can overcome rather unfavorable gradients for Cl^- uptake; freshwater fish are capable of taking up Cl^- from less than 10 μM (150). However, apical proton pump activity appears to drive apical anion exchange, since bafilomycin inhibits Cl^- uptake, by allowing for cellular accumulation of HCO_3^- from CO_2 hydration and possibly by maintaining low HCO_3^- concentrations on the unstirred boundary layer at the apical surface. The observation of electrogenic SLC26a6 operating in an $nHCO_3^-/Cl^-$ mode in marine teleosts (662, 982) offers an interesting perspective for Cl^- uptake by freshwater organisms since such a stoichiometry would benefit from the apical membrane potential and the activity of the electrogenic H^+-pump, and as such would permit Cl^- uptake from freshwater even at low ambient Cl^- concentrations (662). To date, the stoichiometry of freshwater fish SLC26 anion exchangers has not been examined. Exit of Cl^- across the basolateral membrane is believed to occur via a CFTR-like Cl^- channel (1166, 1168, 1696).

Cellular substrate for ion exchange—carbonic anhydrase Aside from NaCl uptake via cotransporters, uptake of both Na^+ and Cl^- requires cellular substrate in the form of H^+ and HCO_3^-, respectively (636). Intracellular carbonic anhydrase (CAII-like, termed CAc in rainbow trout; [599]) is present in MR cells as well as pavement cells (599, 615, 771, 1064, 1497) and plays a role in Na^+ and Cl^- uptake (150, 912, 1429) by facilitating hydration of CO_2 to produce H^+ and HCO_3^-.

MR cell diversity

The morphological subtypes of MR cells first described by Pisam and coworkers (1463, 1464, 1466) show strong resemblance to two subtypes of intercalated cells (α-cells and β-cells) in the mammalian kidney. Peanut lectin agglutinin (PNA) was found to only bind to the β-subtype (β-type) of intercalated cells and has been used successfully to differentiate between these two mammalian kidney cell types (1030). More recently, PNA binding has been employed to differentiate and isolate two types of MR cells (PNA^- and PNA^+, corresponding to α-types and β-types, respectively) from freshwater-acclimated rainbow trout gills (576, 632) and has paved the way for partial physiological characterization of these two cell types (576, 1400, 1402) (Fig. 17). Since these pioneering studies on freshwater rainbow trout, MR cell diversity has been documented from zebrafish and tilapia as recently reviewed (818) (Fig. 17). At least three subtypes of MR cells have been identified in zebrafish: vacuolar H^+-pump rich (HR) cells, Na^+/K^+-ATPase rich (NaR) cells and Na^+:Cl^- cotransporter (NCC) cells (Fig. 17). All three cell types show strong expression of Na^+/K^+-ATPase (818). HR cells are acid-secreting cells displaying Na^+ uptake (501, 800, 801, 1062). Scanning ion-selective electrode techniques (SIET) where employed to demonstrate bafilomycin-sensitive H^+ currents in cells expressing V-type ATPase mRNA and protein (1062). Translational knockdown (morpholinos) of the H^+ pump was found to decrease Na^+ concentrations in HR cells and whole-body zebrafish, providing evidence for a role in Na^+ uptake (501, 800). As discussed above, the quest for an ENaC-like Na^+ channel in fish has been fruitless so far, and indeed, pharmacological and molecular evidence support the involvement of NHEs in zebrafish Na^+ uptake (150, 501). Specifically, NHE3b mRNA is expressed predominantly on HR cells and is upregulated following acclimation to low Na^+ water, implicating this transporter in Na^+ uptake by HR cells (2049). Interestingly, NHE2 and NHE3 have been documented to localize to trout PNA-positive cells (835). The presence of NHE3b and the H^+ pump in the same cell type may seem counterintuitive, but it appears from manipulations of ambient pH and Na^+ concentrations that NHE3b is primarily regulated based on Na^+ availability while the H^+ pump is regulated by ambient pH (2049) suggesting distinct roles for these two transporters. The anion exchangers (SLC26a3, a4 and a6) recently implicated in Cl^- uptake by zebrafish (81, 1430) have yet to be localized to specific MR cells. However, the apparent dependence on H^+ extrusion (150) points to a role for HR cells in Cl^- uptake via anion exchange.

Not surprisingly, HR cells express an apical, extracellular membrane-associated carbonic anhydrase (CA)15a isoform and cytosolic CA2 (equivalent to trout CAc), and pharmacological manipulations as well as morpholino experiments have demonstrated a role for acid excretion as well as Na^+ uptake (150, 501, 1064). In agreement with these observations, trout CAc has been demonstrated to localize to MR cells in trout gills and to play a role in acid-base regulation (599). NaR cells show strong expression of Na^+/K^+-ATPase but appear to be primarily involved in Ca^{2+} uptake via apical epithelial calcium channels (ECaC), basolateral Ca^{2+} ATPase and the Na^+/Ca^{2+} exchanger (NCX) (1047, 1396). The activity of the Na^+/K^+-ATPase likely provides conditions for the operation of NCX. NCC cells express a Na^+:Cl^- cotransporter isoform, and loss-of-function experiments (morpholinos) showed significant decreases in Cl^- uptake and Cl^- content of embryo

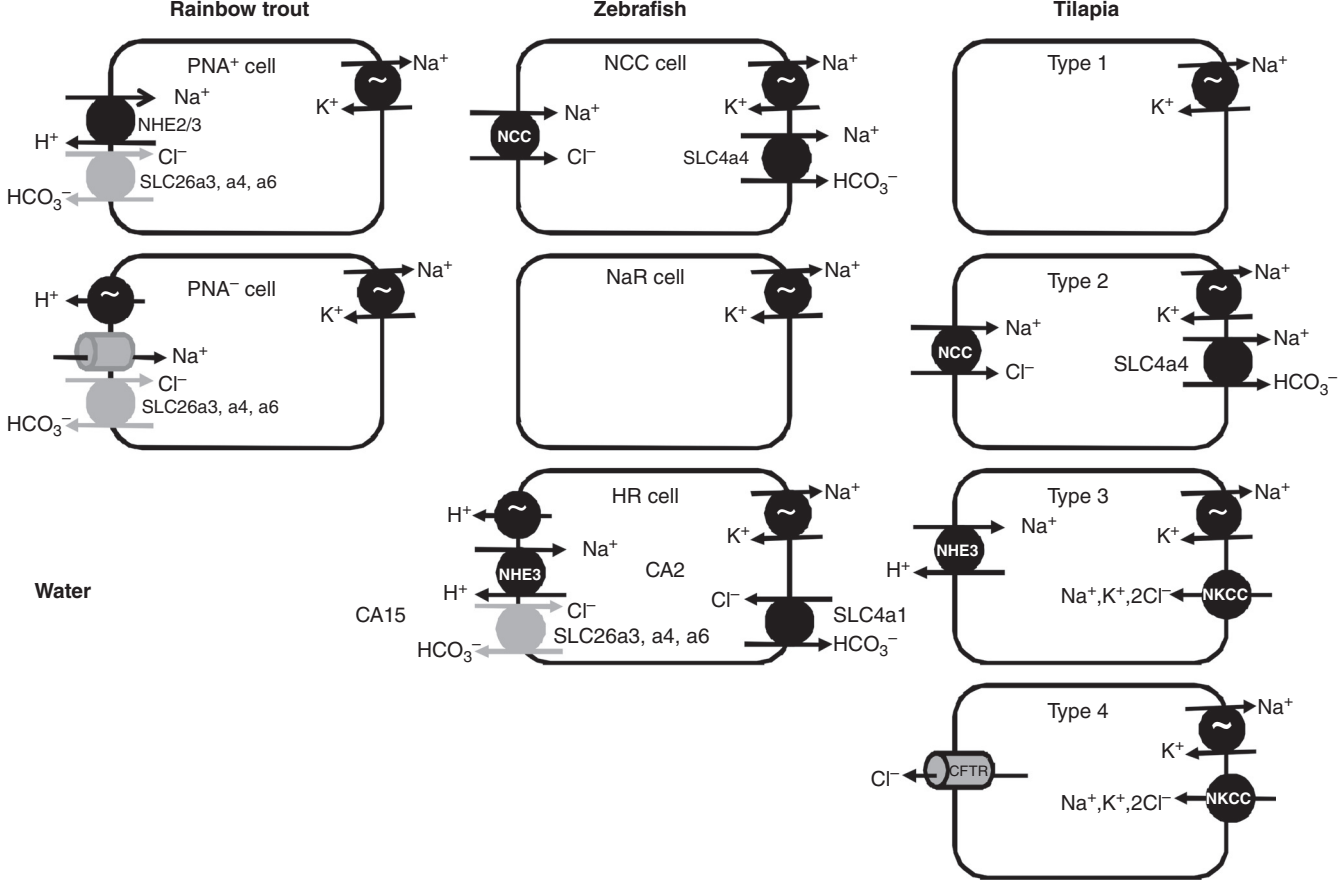

Figure 17 Schematic representation of accepted (black) and putative (gray) transporters and processes in freshwater fish gill MR cell types (modified from [818]). Classification of cell types is based in part on immunohistochemistry and in part of transport properties of individual cells as assessed by ion-selective electrodes or isolation of individual cell types. All MR cells express high levels of basolateral Na^+/K^+-ATPase. Rainbow trout PNA^+ cells express apical NHE isoforms while the PNA^- cells express apical H^+-pumps and a Na^+-channel yet to be identified. Both PNA^+ and PNA^- cells take up Cl^- via Cl^-/HCO_3^- exchange processes (1402), and although the nature of the anion exchanger(s) involved are unknown, members of the SLC26 family are likely candidates. Zebrafish possess at least three distinct MR cell types. NCC cells are characterized by apical an Na^+:Cl^- cotransporter (NCC) and a basolateral Na^+:HCO_3^- cotransporter (SLC4a4) (1028). The NaR cell type, in addition to high levels of Na^+/K^+-ATPase, contains apical and basolateral Ca^{2+} transporters (not shown) and is likely involved in Ca^{2+} acquisition. The third zebrafish cell type, HR, shows strong expression of apical H^+-pumps and expression of apical NHE3 as well as basolateral SLC4a1 (1028). Furthermore, cytosolic carbonic anhydrase (CA2) and membrane bound extracellular carbonic anhydrase (CA15) in HR cells are involved in ion uptake. Uptake of Cl^- by zebrafish occurs via three members of the SLC26 gene family which have yet to be localized to specific cell types. However, due to the dependence of Cl^- uptake on H^+-pump activity, the SLC26 transporters have been indicated in NR cells in the present representation. The euryhaline tilapia acclimated to freshwater have as many as four unique MR cell types. So far, type 1 cells have only been demonstrated to express basolateral NKA, and their function remains to be elucidated. Tilapia cell type 2 appears identical to zebrafish NCC cells, with apical NCC and basolateral SLC4a4 cotransport, while cell type 3 express apical NHE3 and basolateral NKCC (presumably NKCC1). Basolateral NKCC(1) is also found in tilapia cell type 4, which also express apical CFTR Cl^- channels. It should be noted that the representations illustrate what currently is supported by the peer-reviewed literature, and that multiple additional transporters and enzyme not included in these diagrams must also be present to account for the observed transport properties of the freshwater teleosts fish gill.

morphants (1930). Multiple types of MR cell are seen also in tilapia embryonic skin, which display types I, II, III and IV (Fig. 17), classified by differential labeling for different transporters (773, 829). All four subtypes display strong basolateral staining for Na^+/K^+-ATPase. To date, no other transporters have been identified in Type I cells. Type II cells show apical NCC localization and are analogous to the zebrafish NCC cells (818), while Type III cells show basolateral NKCC and apical NHE3 staining and seems analogous to HA cells from zebrafish. Type IV cells from tilapia held in freshwater appears to be a seawater-type MR cell (see below) with basolateral NKCC and apical CFTR staining. Experiments allowing fish to acclimate to distinct water chemistries suggested that Type II cells are involved in Na^+ and Cl^- uptake and that type III cells are contributing to Na^+ uptake, while Type IV is responsible for Na^+ and Cl^- secretion (773, 829, 830).

Gill epithelial permeability

Much focus has been placed on the active Na^+ and Cl^- uptake pathways in freshwater fish, with less attention directed

toward potential control of the passive branchial efflux component. A high number of studies have demonstrated substantial differences in trans-gill epithelial potential (TEP) among euryhaline fish, depending on exposure to different ambient salinities, and furthermore, that these differences are attenuated by salinity acclimation (reviewed by [1167]). More recently, a detailed time course revealed that TEP changes gradually over a 12-30-hr period after transfer of seawater acclimated killifish (*Fundulus heteroclitus*) to freshwater, settling at levels representative of fully freshwater acclimated individuals (2025, 2027). These observations were interpreted as changes in the relative permeability of Cl^- compared to Na^+ (P_{Cl}/P_{Na}), probably associated with closing of the paracellular Na^+ shunt pathway (2025). Indeed, relatively rapid reductions in Na^+ efflux have been reported for fish exposed to reduced ambient Na^+ concentrations (200, 695), while a single study has demonstrated similar responses for Cl^- (1428). Changes in gill permeability are thought to be related, in part, to altered lipid bilayer composition (695, 696), and more recently the tight junction proteins, occludin and claudins, have been implicated in the control of gill epithelial permeability. Occludin is a four-transmembrane protein exhibiting a physiological role in determining the barrier properties of vertebrate epithelia (521, 624), and recently documented to be present in fish epithelia, including the gill (274-277). Occludin expression was elevated in goldfish following transfer to ion-poor water (274), and occludin protein levels in rainbow trout gill epithelial cultures are inversely correlated with epithelial permeability (277). These observations point to a role for occludin in controlling epithelial barrier functions in freshwater fish gills.

Like occludin, claudins are four-transmembrane domain proteins and integral components of tight junctions (1879). The physiology of claudins is an emerging field complicated by their diversity; at least 24 claudins are known from mammals and no less than 56 are found in the puffer fish, *Fugu rubripes* (1091, 1901). Nevertheless, evidence so far suggests that a number of claudins (claudins 3, 3a, 3c, 8d, 27a, 27b, 28a, 30) may be upregulated in gills of euryhaline fish (tilapia, salmon, puffer fish and eels) acclimated to freshwater, where they play a role in tightening the branchial epithelium (58, 59, 459, 879, 1832, 1834, 1835).

Gastrointestinal tract

By far the majority of studies of osmoregulation by freshwater fish have been performed on fasted animals to avoid fouling of the water in closed systems most commonly used for ion uptake measurements. Consequently, little is known about the dietary contribution to osmoregulation by freshwater fish. However, the question of potential dietary contributions to salt homeostasis in freshwater fish has received some attention in the past few years (216, 217, 2024); the field has been summarized and synthesized recently (2023). Natural diets for fish, consisting of aquatic invertebrates, contain 34-41 mmol Na^+/Kg (947). Assuming a food consumption rate of 3% body mass per day and complete assimilation of dietary Na^+, dietary intake would amount to 20-24% of the branchial Na^+ uptake rates of a standard 100-gram fish. Consistent with this relatively modest predicted contribution are observations of no net Na^+ gain from ingested diets in freshwater acclimated rainbow trout (216). However, it should be noted that dietary Na^+ uptake may play a more significant role in freshwaters of low ionic strength where branchial uptake maybe limited. For example, at ambient Na^+ concentrations, branchial Na^+ uptake by the freshwater stingray (*Potamotrygon sp.*) is insufficient to offset the diffusive Na^+ loss (2029). The obvious conclusion is that dietary Na^+ uptake is of quantitative significance for salt balance in this freshwater elasmobranch.

In contrast to the low Na^+ absorption observed in rainbow trout, 89% and 81% of ingested K^+ and Cl^-, respectively, was assimilated by the same fish suggesting that dietary Cl^- and especially K^+ may serve an important source of these electrolytes to freshwater fish (216). The pathway for dietary K^+ uptake in freshwater fish is unknown, but Cl^- uptake from the diet appears to occur in exchange for HCO_3^- secretion (215, 338, 1816), which acts to neutralize acidic chyme from the stomach and alleviate, or at least reduce, the alkaline tide in fish compared to that seen in many postprandial terrestrial vertebrates (1321).

Kidney and urinary bladder

Due to the diffusive water gain mainly across the gill surface, urine flow rates are generally high in freshwater fish ranging between 2 ml/kg/h and 10 ml/kg/h (193, 366, 668, 749, 1207). Tubular reabsorption of filtered ions is efficient such that the dominant electrolytes, Na^+ and Cl^-, are typically less that 5-10 mM with other electrolytes being in the submillimolar range (749). Thus, net renal Na^+ loss is in the range of 10-100 nmol/gram/hour, which has led to the common perception that renal NaCl handling is of minor significance for osmoregulation. However, considering a glomerular filtration rate of 5 ml/kg/hour and a plasma Na^+ concentration of 150 mM, renal Na^+ filtration occurs at a rate of 750 nmol/gram/hour. More than 90% (668), or 675 nmol/gram/hour, of the filtered Na^+ is reabsorbed by the renal tubules, which compares to branchial Na^+ uptake rates of 210 nmol/gram/hour in a standard 100-gram fish. Thus, renal transport processes are highly significant for freshwater fish volume control as well as ion regulation.

Tubule anatomy In contrast to marine conspecifics, freshwater fish kidneys have distal renal tubules connecting proximal segments to the collecting duct via a narrow, short intermediate segment and collecting tubules (749). With very few exceptions, freshwater fish have glomeruli contained in renal corpsules (1167). At least three evolutionary events have led to the loss of glomeruli, presumably in species inhabiting marine environments, but aglomerular fish are found in freshwater (115, 329, 330). Obviously, urine production in these

species occurs strictly by secretion as discussed for marine teleost kidneys below.

Glomerular filtration and urine flow Urine formation in freshwater fish consists of glomerular ultrafiltration and substantial reabsorption of mainly monovalent ions across the tubular epithelia. The tubular epithelium of freshwater teleost kidneys exhibits low water permeability, with the lowest permeability seen in the most distal regions. However, some water is absorbed with the reabsorption of electrolytes and organic solutes such that urine flow rates generally are lower than glomerular filtration rates (749). In contrast to the situation for terrestrial vertebrates that display regulated tubular water reabsorption, urine flow rates in freshwater teleosts are directly proportional to glomerular filtration regardless of metabolic rate (748, 749, 784). In contrast to mammals, fish display glomerular intermittency, and whole kidney glomerular filtration is the product of all-or-none regulation of individual nephrons (193, 208, 749).

Tubular reabsorption

As in mammals, some reabsorption of electrolytes occurs in the proximal tubules, which are also the site for glucose and HCO_3^- reabsorption (218, 598). However, the majority of ion absorption by freshwater fish tubules occurs in the water-impermeable distal tubules (193). In the proximal tubule, basolateral Na^+/K^+-ATPase drives Na^+ uptake via apical Na^+ glucose, Na^+ amino acid transporters and NHEs. Absorption of Cl^- by the proximal tubule occurs via electro-neutral anion exchange coupled to the function of NHEs (193). Absorption of Na^+ and Cl^- in the early distal tubules occurs in part via NKCC2 while some paracellular Na^+ transport driven by the serosal negative potential may also occur. The operation of NKCC may rely on recycling of K^+ across the apical membrane via K^+ channels. Transport of Cl^- across the apical membrane occurs via K^+:Cl^- cotransporters or conductive Cl^- channels (193, 384, 1320). The reabsorption processes in the late distal tubules, collecting tubules and collecting ducts of teleosts remain to be characterized.

Role of the urinary bladder

The urinary bladder forms a final diluting segment comprised of a high resistance, tight epithelium with exceedingly low water permeability and capable of net absorption down to about 2 mM NaCl (1162). In salmonids, Na^+ and Cl^- uptake is electrically silent and largely independent of the counterion (225, 1161). NaCl absorption is sensitive to amiloride but not affected by thiazide and bumetanide. Furthermore, Na^+ uptake in absence of Cl^- is associated with acidification of the luminal solutions (225, 1164, 1166). These observations all points to ion absorption via NHE and anion exchangers.

Renal function and ambient salt concentrations

Given the quantitatively significant role of tubular NaCl absorption for ion regulation by freshwater fish, the paucity of data on the response of renal Na^+ and Cl^- transporters to alterations of ambient NaCl concentrations is striking, especially considering the substantial effort devoted to branchial transport processes. Renal transport processes in freshwater fish are clearly subject to regulation depending on acid-base status (598) and Na^+ (668) balance, but nothing is known about the nature of this regulation.

Marine Teleost Fish

Overview

Marine teleosts, being hypoosmotic with respect to the environment, experience a continuous diffusive water loss and ion gain across the gill epithelium. Although urine flow is modest in marine teleosts when compared to freshwater species, fluids lost both by urine and across the gill epithelium dictate a need for ingestion of seawater to maintain water balance (651). The gastrointestinal tract is capable of water absorption from ingested seawater, a process that ensures water balance but leads to additional salt gain and thus a need for excretion of mainly Na^+ and Cl^-, but also Mg^{2+} and SO_4^{2-}. Since fish kidneys are incapable of producing hyperosmotic urine, monovalent ions are excreted mainly across the gill while renal excretion of low urine volumes compensates for excess Mg^{2+} and SO_4^{2-} gain (1167).

Gills

Early studies identified "chloride cells" (now referred to as MR cells) as the likely site of Cl^- excretion by gills of seawater-acclimated European eel (913, 914). More recent studies, using the opercular epithelium of the killifish as a model for marine fish gills, demonstrate that density of MR cells and epithelial conductance is proportional to ambient salinity and that the MR cells indeed are responsible for Cl^- secretion (370, 545). In seawater, pavement cells cover ~90% of the gill surface area, with MR cells occupying mainly the trailing edge of the filament and the interlamellar regions (726, 890). In addition to numerous mitochondria and an extensive tubular network derived from basolateral invaginations, branchial MR cells of marine teleosts are characterized by an apical crypt and are always associated with adjacent accessory cells (890, 1461). MR cells and accessory cells show extensive interdigitations, have shallow tight junctions and are considered to be the site of the relatively high ionic permeability of the marine teleost gill epithelium (509, 884, 1606).

Na^+ and Cl^- excretion The transport model for marine teleost fish Na^+ and Cl^- excretion (Fig. 18) is better established than the models for Na^+ and Cl^- uptake by freshwater fish. Excretion of Na^+ and Cl^- is driven by Na^+/K^+-ATPase,

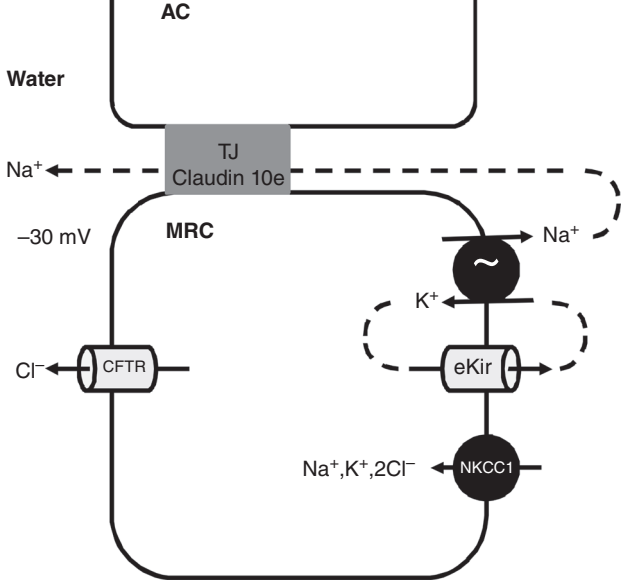

Figure 18 Accepted transport processes in the branchial epithelium of marine teleost fish. MRC = MR cell, AC = accessory cell. Transcellular Cl^- secretion includes entry across the basolateral membrane by the secretory isoform of the Na^+-$2Cl^-$-K^+ isoform (NKCC), which is driven by the electrochemical Na^+ gradient established by the Na^+/K^+-ATPase (~), and exit across the apical membrane via the cystic fibrosis transmembrane regulator (CFTR) Cl^- channel. The active secretion of Cl^- establishes a transepithelial potential of −17-40 mV (reference blood side), which drives paracellular Na^+ secretion through cation-selective "leaky junctions" between MRCs and ACs. Cation selectivity of the junctions may be mediated by claudin 10e and possibly other claudins. A basolateral inward rectifying K^+ channel (eKir) allows for recycling across the basolateral membrane. The depicted transport model also described Na^+ and Cl^- secretion by the renal proximal tubules and the elasmobranch rectal gland. See the text for further details.

which is highly abundant in the basolateral membrane (885, 2004, 2006), as ouabain inhibits not only enzyme activity but also efflux of both ions (1417, 1690). The basolateral Na^+/K^+-ATPase establishes a Na^+ gradient allowing for entry of Cl^- and Na^+ via the basolateral secretory Na^+-$2Cl^-$-K^+ cotransporter (NKCC1), for which the gene is expressed in proportion to ambient salinity (367, 1098, 1168, 1417, 1836) leading to corresponding protein levels (538, 882, 1605). Observations of Ba^{2+} blocking the short-circuit current across killifish opercular epithelia (413), the cloning of an inward-rectifying K^+ channel (Kir) from Japanese eel and observations of increased expression of Kir during acclimation to seawater (1793) suggest that K^+ is recycled across the basolateral membrane, entering via NKCC1 and exiting via Kir, to allow for Cl^- and Na^+ uptake. Once in the MR cells, Cl^- is above thermodynamic equilibrium and exits across the apical membrane via a CFTR-type channel. Teleost CFTR was first identified in killifish (1165, 1696) and has since been identified in a number of seawater teleosts. Gene expression (149, 1835) and CFTR protein amount (1662, 1805, 1871) increase with increasing salinity. In addition to transcriptional regulation, CFTR function, at least in the euryhaline killifish, is subject to translocation of protein from cytosolic compartments to the apical membrane (1168) and is under regulation by phosphorylation of focal adhesion kinase (1169).

Cation selectivity of gills and opercular epithelia from seawater-acclimated trout and killifish, respectively, provided evidence that Na^+ secretion across marine fish gill occurs passively via a paracellular shunt pathway (414, 937, 1163). The magnitude of the gill transepithelial potential (TEP) in most seawater teleosts ranges from −17 to −40 mV (reference in body fluids) (1167), which in many cases is insufficient to drive Na^+ secretion against the blood:water concentration gradient. However, it is likely that the TEP measured *in vivo* is less than that localized at the MR cells due to the large surface area of pavement cells, which adds to the passive shunt (1167). The gulf toadfish represents an exception by having an outside positive gill TEP (506), suggesting unique ion permeability of the gill and that Na^+ secretion by this species may not be passive.

To date, a single member of the claudin gene family, claudin 10e of Atlantic salmon, shows increased branchial expression following seawater transfer (1835), pointing to a role of this tight junction protein in controlling the cation permeability of the seawater acclimated fish gill.

MR cell diversity Like freshwater teleosts, marine teleosts may possess distinct MR cell types; at least one marine teleost has been reported to have one cell type expressing apical NHE2 and basolateral Na^+/K^+-ATPase, while another expresses basolateral H^+-ATPase, and are likely involved in acid and base secretion, respectively (263).

Gastrointestinal tract

Seawater ingestion and intestinal water absorption was demonstrated in 1930 (1713), and it has since been established that drinking rates in marine and seawater-acclimated euryhaline fish range from 1 to 5 ml/kg/h (254, 564-566, 568, 660, 663, 1061, 1663, 1800), 10-50-fold higher than in freshwater fish (254, 1061, 1148, 1423). The majority (70%-85%) of ingested seawater is absorbed by the intestine with the remainder being excreted as rectal fluids (597, 747, 1663, 2009). Gastrointestinal processes, rather than branchial and renal processes, involved in osmoregulation have recently been demonstrated to be limiting for survival of teleost fish in high-saline environments (596).

Control of drinking rates

Changes in drinking rates present the most immediate level of control over fluid absorption by the gastrointestinal tract, and control of the drinking rate is therefore central to osmoregulation by marine fish. Control of teleost fish drinking rates have been reviewed recently (654, 1798) and includes a rapid response to changes in ambient salinity, distension of the gastric wall and intestine, as well as luminal salt concentrations (42, 769). Although it cannot be excluded that these rapid responses are mediated by the renin-angiotensin system

(RAS, discussed below), it occurs within minutes and much faster than the onset of significant hypotension or hypovolemia caused by dehydration (70). Ion replacement studies demonstrated that Cl^- is the trigger for externally stimulated drinking and the negative feedback resulting from elevated luminal Cl^- concentrations in the intestinal lumen (42, 769). RAS is clearly involved in regulation of drinking by teleosts via angiotensin II (ANG II) acting on the so-called swallowing center in the medulla oblongata to initiate water ingestion (254, 563, 565, 1423, 1797, 1799, 1800). Hypovolemia and hypotension as a result of vasodilation potently stimulate RAS-induced drinking via elevated ANG II (565, 769). In addition, bradykinin, the active component of the kallikrein-kini system, and atrial natriuretic peptide both act on RAS by altering ANG II levels to inhibit drinking, although it is unknown whether these effects are mediated solely through RAS (334, 335, 585, 1801, 1872).

Esophageal desalinization

Around 50% of NaCl ingested with seawater is absorbed by the relatively short esophagus in a $1Na^+$:$1Cl^-$ ratio (651). This high NaCl absorption rate, combined with low water permeability (770, 1403), explains the marked reduction in osmotic pressure of ingested seawater (~1000 mOsm) as it travels to the gastric lumen (~500 mOsm) (930, 1403, 1713, 2009). Passive and active absorption of Na^+ and Cl^- occur in the esophagus, but it appears that the esophagus is relatively impermeable to other ions and water. For the few species examined, Na^+ uptake occurs via Na^+/H^+ exchange or Na^+ channels (amiloride sensitive) rather than NCC or NKCC2 cotransporters (770, 1403), while the mechanisms of Cl^- uptake remain unknown.

Absorption by the intestine

Intestinal water absorption in marine teleosts is tightly linked to absorption of Na^+ and Cl^- (1123, 1701, 1888) with >95% of the ingested NaCl being absorbed (597). The absorbate is hyperosmotic (651) and the resulting net salt gain is compensated by branchial excretion (discussed above). Ion absorption in the anterior segment of the intestine results in isoosmotic fluids in the intestinal lumen, allowing for solute coupled water absorption (651, 654, 662, 665). Entry of Na^+ across the apical membrane occurs via two cotransport pathways, NKCC2 and NCC (367, 530, 560, 687, 688, 1266) (Fig. 19). Considering the Na^+ and H^+ gradients across the apical membrane, both Na^+-channels and NHEs may contribute to Na^+ uptake from the lumen, but at present no evidence has been reported for involvement of Na^+ channels. However, elevated NHE3 mRNA expression in rainbow trout intestinal mucosa during acclimation to seawater (658) suggests a role for this NHE in intestinal Na^+ absorption, although luminal amiloride in the pyloric caecae and anterior regions does not appear to reduce H^+ secretion (657). A role for NHEs in intestinal Na^+ absorption was also suggested by *in situ* gut perfusion experiments on seawater-acclimated rainbow trout showing reduced acid-secretion in the presence of luminal amiloride (2009).

Transport of Na^+ across the basolateral membrane occurs via Na^+/K^+-ATPase that is highly abundant in the basal and lateral membranes of the columnar enterocytes (653). The intestinal epithelium shows the highest Na^+/K^+-ATPase activity of the three osmoregulatory organs (655, 786) and mRNA expression of the α-subunit of the pump, as well as enzymatic activity is most often higher in seawater-acclimated compared to freshwater-acclimated euryhaline species—a trend that also applies to NKCC2 mRNA levels (328, 567, 838, 909, 1141, 1142, 1644, 1836). In addition to fueling apical entry of Na^+, the Na^+/K^+-ATPase also energizes Na^+ transport processes across the basolateral membrane. Basolateral H^+ extrusion occurs via a Na^+-dependent mechanism, presumably NHE1 (595, 656), and basolateral Na^+:HCO_3^- cotransport is important for high rates of intestinal HCO_3^- secretion (see below; [658, 1813]).

The intestine displays net K^+ absorption. Considering K^+ concentrations in seawater and the intestinal fluids are 10 and ~5 mM, respectively (661), and the substantial water absorption, it is clear that the majority of the ingested K^+ is absorbed. Undoubtedly, NKCC2 is involved in K^+ absorption, but considering the concentrations of K^+, Na^+ and Cl^- in seawater, it becomes clear that K^+ might limit NKCC2 function. Observations of a Ba^{3+}-sensitive K^+ channel (1266) suggests that K^+ may recycle across the apical membrane to facilitate transport by NKCC2 as is seen for NKCC1 in the basolateral membrane of the marine teleost gill epithelium (see above). A basolateral K^+:Cl^- cotransporter facilitates K^+ movement across the basolateral membrane (1711).

The net absorption of Cl^-, like Na^+, occurs against an electrochemical gradient (Table 6 and Fig. 19). However, in contrast to Na^+, which enters across the apical membrane down an electrochemical gradient, Cl^- uptake across the apical membrane occurs against an electrochemical gradient. Cl^- movement from the cytosol across the basolateral membrane is following the electrochemical gradient via K^+:Cl^- cotransport or Cl^--channels, including a CFTR paralogue (1097, 1168, 1711). At least two distinct pathways contribute to apical Cl^- entry in the marine teleost intestinal epithelium. One pathway relies on the inward-directed electrochemical Na^+ gradient via cotransporters (NCC and NKCC2) discussed above, while the other pathway is via Cl^-/HCO_3^- exchange. The evidence for the presence of these two parallel pathways, which are both quantitatively important, has been discussed in detail in previous reviews (651-654, 661, 662, 665). High HCO_3^- concentrations in intestinal fluids were first inferred in 1930 (1713) and have since been documented for a high number of marine or seawater-acclimated euryhaline fish (651, 661, 1814, 2008), and are the result of high-activity apical Cl^-/HCO_3^- exchange proteins in the apical membrane. Convincing correlations between Cl^- uptake and apparent HCO_3^- secretion across the intestinal epithelium of a seawater-acclimated teleost was first reported by Shehadeh and Gordon (1663) and was attributed to anion exchange,

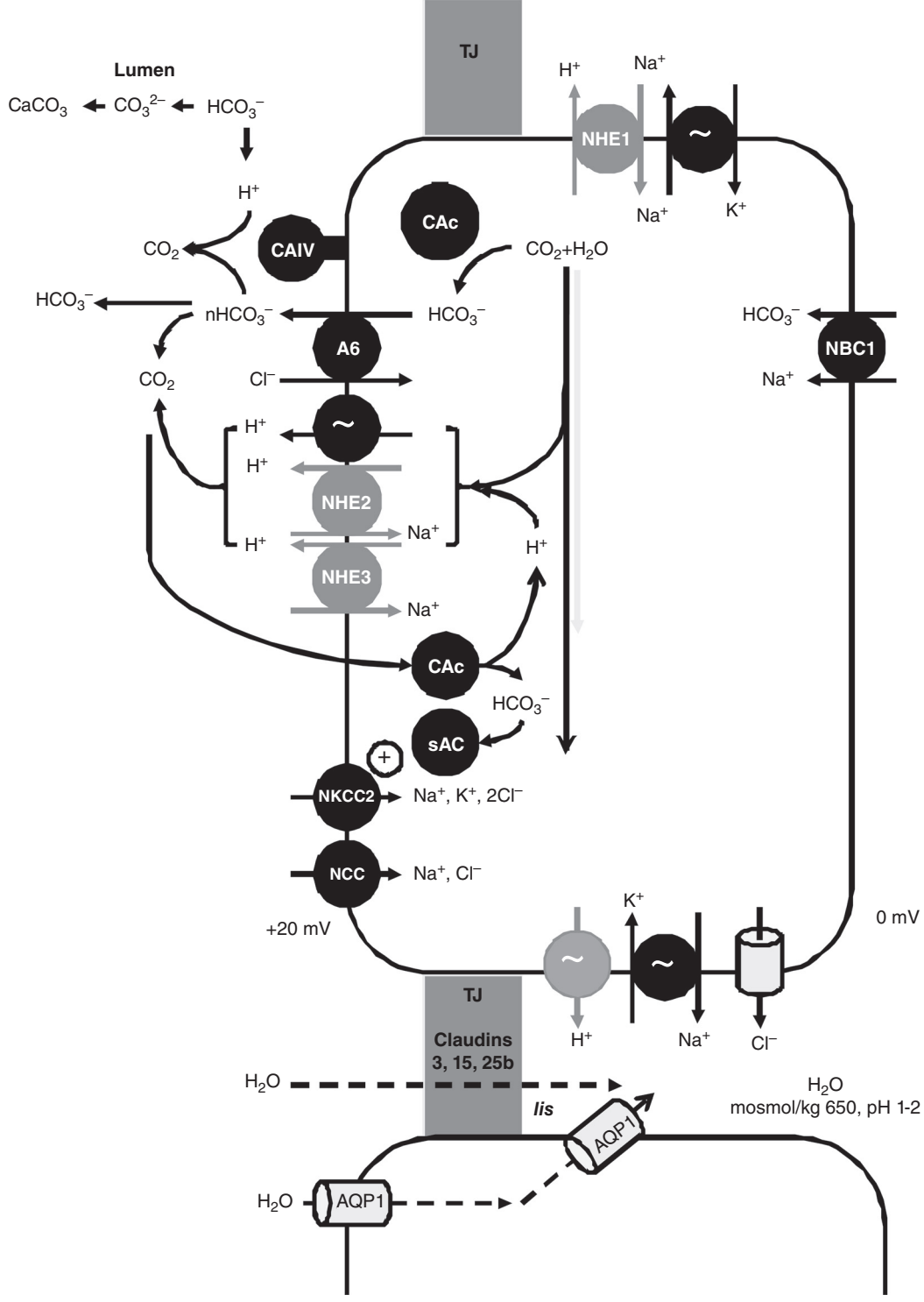

Figure 19 Accepted (solid black) and putative (gray) transport processes in the intestinal epithelium of marine teleost fish (redrawn from [652, 654, 662]). Water transport, transcellular (potentially via AQP1) and/or paracellular (dotted lines) is driven by active NaCl absorption providing a hyperosmotic coupling compartment in the lateral interspace (lis). Entry of Na+ across the apical membrane via cotransporters (NKCC2 and NCC) and extrusion across the basolateral membrane via Na+/K+-ATPase accounts for transepithelial Na+ movement. In addition, recent evidence that NHE2 and NHE3 are expressed in the intestinal epithelium suggested that Na+ uptake across the apical membrane may occur also via these transporters. Entry of Cl^- across the apical membrane occurs via both cotransporters and Cl^-/HCO_3^- exchange conducted by the SLC26a6 anion exchanger while Cl^- exits the cell via basolateral anion channels. Cellular HCO_3^- for apical anion exchange is provided in part by HCO_3^- entry across the basolateral membrane via NBC1 and in part by hydration of endogenous metabolic CO_2. Cytosolic carbonic anhydrase (CAc) found mainly in the apical region of the enterocytes facilitates CO_2 hydration. (*Continued*)

which accounts for continued Cl^- uptake in absence of luminal Na^+ (655, 661). Recent reports identified at least one anion exchange protein, SLC26a6, to be important for Cl^-/HCO_3^- exchange across the apical membrane (662, 982). Interestingly, this anion exchanger operates in a $Cl^-/nHCO_3^-$ exchange mode, is therefore electrogenic and is fueled in part by the apical membrane potential to allow for transport of Cl^- and HCO_3^- against seemingly unfavorable chemical gradients (662).

The salt absorption described above facilitates water absorption despite the lack of net osmotic gradients. The absorbate is significantly hypertonic (651), and it appears that the lateral interspace (*lis*) between enterocytes acts as a coupling compartment for salt and water absorption (Fig 19). Salt transport across the lateral membranes renders the fluids in *lis* hyperosmotic, which drives water movement. Water absorption in the direction from the lumen to the blood is likely ensured by barrier properties of the tight junctions, resulting in dispersal of salts and thereby water in the direction from *lis* to the blood side of the epithelium. See recent reports tor detailed accounts of solute-coupled water movement across vertebrate epithelia (997-1000, 1301). It is unknown whether water enters the *lis* from the intestinal lumen across the tight junctions or by a transcellular pathway, although a recent study points to water movement mainly by a transcellular route (2026). Of the three aquaporins (AQPe, AQP1 and AQP3; [49, 368, 1051, 1176, 1177, 1500]) observed to be expressed in the marine teleost intestine, AQP1 may play a role in water absorption. Observations of elevated mRNA expression following seawater transfer, elevated AQP1 protein abundance in intestinal tissue following seawater acclimation and finally apical as well as basolateral localization of the protein suggests a role in transepithelial water movement (49, 1176, 1177, 1500). It has been demonstrated that AQP1 confers water permeability in expression systems, but despite the above observations, unequivocal evidence for a role for AQP1 in intestinal water absorption is lacking.

Exclusion and secretion by the intestine

Luminal concentrations of Mg^{2+} and SO_4^{2-} are typically 120-150 mM and 100-120 mM, respectively, due to selective absorption of Na^+, Cl^- and water (Table 6 and [596, 597, 651, 661, 1167]). As plasma concentrations of these divalent ions are < 1 mM, the gradient from the intestinal lumen to the extracellular fluids is greater than it is for any other ions. With this in mind, it is remarkable that ~90% of the ingested $MgSO_4$ passes through the intestinal tract unabsorbed (597, 747, 2010). Nevertheless, some Mg^{2+} and SO_4^{2-} are absorbed owing to the considerable gradient across the intestinal (and branchial) epithelium. The resulting excess of these divalent ions is cleared by renal excretion, as discussed below. Limited intestinal SO_4^{2-} absorption is not strictly a matter of barrier functions as SO_4^{2-} secretion via anion exchange for Cl^- uptake acts to reduce overall net SO_4^{2-} uptake (1415). The nature of the anion exchanger responsible for intestinal SO_4^{2-} secretion remains unknown, but recent studies demonstrated that SLC26a6 is responsible for SO_4^{2-}/Cl^- exchange by renal tubules of marine teleosts (889). Since urine and intestinal fluids of marine teleosts are similar with respect to Cl^- and SO_4^{2-} concentrations (Table 6), it is possible that SLC26a6 acts to secrete SO_4^{2-} as well as HCO_3^- in exchange for Cl^- uptake. The situation is even less clear for Mg^{2+} as it is unknown whether the substantial gradients are maintained strictly by barrier functions or if intestinal Mg^{2+} secretion contributes to the very low net uptake. Recent findings show changes in salinity alter mRNA expression of multiple isoforms for claudin 3, 15 and 25b in the intestinal epithelium of euryhaline fish (308, 1838), which suggests that these claudins may be involved in controlling and regulating the paracellular pathway and thus potentially the barrier functions of the epithelium against movement of divalent ions. Of particular interest is that certain isoforms are expressed more robustly in the distal segments (308) where luminal-serosal gradients are the steepest (1167).

In contrast to Mg^{2+} and SO_4^{2-}, Ca^{2+} concentrations are typically low (<5 mM) in intestinal fluids of marine fish. However, these low concentrations are not due solely to intestinal Ca^{2+} uptake but are the product of $CaCO_3$ precipitate formation facilitated by the high luminal HCO_3^- concentrations and alkaline conditions (2010, 2012). Precipitate formation is a consistent phenomenon among marine fish and has recently been demonstrated to contribute significantly to oceanic $CaCO_3$ production (2011). Although the fraction of

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Figure 19 (*Continued*) Protons arising from CO_2 hydration are extruded mainly across the basolateral membrane by a Na+-dependent pathway and possibly by vacuolar H+ pumps. Some H+ extrusion occurs across the apical membrane via H+ pumps and possibly via NHE2 and/or NHE3 and masks some of the apical HCO_3^- secretion by HCO_3^- dehydration in the intestinal lumen yielding molecular CO_2. This molecular CO_2 may diffuse back into the enterocytes for rehydration and continued apical anion exchange. Furthermore, molecular CO_2 from this reaction is rehydrated in the enterocytes and resulting HCO_3^- is sensed by soluble adenylyl cyclase (sAC), which appears to stimulate ion absorption via NKCC2 (+)(1859). Conversion of HCO_3^- to CO_2 in the intestinal lumen is facilitated by membrane-bound carbonic anhydrase, CAIV and possibly other isoforms, a process that consumes H+ and thereby contributes to luminal alkalinization and CO_2^- formation. Titration of luminal HCO_3^- and formation of CO_2^- facilitates formation of $CaCO_3$ precipitates to reduce luminal osmotic pressure and thus aid water absorption. SLC26a6, the electrogenic anion exchanger, exports $nHCO_3^-$ in exchange for $1Cl^-$, and its activity is therefore stimulated by the hyperpolarizing effect of the H+ pump. The apical electrogenic $nHCO_3^-/Cl^-$ exchanger (SLC26a6) and electrogenic H+ pump constitutes a transport metabolon, perhaps accounting for the apparently active secretion of HCO_3^- and the uphill movement of Cl^- across the apical membrane. The indicated values for osmotic pressure and pH in the absorbed fluids are based on measured net movements of H_2O and electrolytes including H+s, but the degree of hypertonicity and acidity in lis likely are much less than indicated due to rapid equilibration with subepithelial fluid compartments. "TJ" = tight junction. Selective permeability of the epithelium is likely related to multiple isoforms of claudin 3, clauding 15 and claudin 25b, although other claudins may also be involved. See text for further details.

ingested Ca^{2+} taken up by the intestinal epithelium differs among reports and possibly species, the formation of precipitates in the intestinal lumen acts to reduce osmotic pressure by up to 70 mOsm as Ca^{2+} and HCO_3^- are removed from solution. A reduction of osmotic pressure of this magnitude is significant for water absorption and thus osmoregulation (1983, 2012). Facilitation of $CaCO_3$ formation in the intestinal lumen is possibly enhanced by the activity of an extracellular, apical-membrane-bound carbonic anhydrase isoform (possibly CA-IV) (657, 658).

Secretion of HCO_3^- by the teleost intestine correlates with ambient salinity and is temperature dependent (656) but is generally in the order of 0.5 μmol/cm^2/h under resting, physiological conditions (43, 436, 570, 656, 657, 661, 662, 667, 1813, 1815, 2012). This rate is comparable to resting secretion rates by the mammalian duodenum at 37°C (301, 785, 1877, 1878); the high rates of HCO_3^- secretion lead to luminal concentrations of up to 100 mM (651, 662, 2012).

Transepithelial HCO_3^- transport as well as hydration of endogenous CO_2 within the intestinal epithelium both contribute to the high rates of secretion, although to varying extent in different species (43, 436, 656, 657, 667, 2010). Considering transepithelial transport, entry of HCO_3^-across the basolateral membrane takes place via an electrogenic Na^+:HCO_3^- cotransporter (NBC1), for which expression is dependent on ambient salinity (982, 1813). Intestinal cytosolic carbonic anhydrase (CAc) catalyzes the hydration of endogenous CO_2 providing HCO_3^- for apical anion exchange; CAc mRNA expression in intestinal tissues also shows elevated mRNA expression following transfer to elevated salinity (658, 1610). CAc localizes to the apical region of the intestinal epithelium (658), and application of permeant CA inhibitors inhibits luminal HCO_3^- secretion (436, 657, 667, 2009), demonstrating an important role for this enzyme in apical anion exchange. Intestinal HCO_3^- secretion in the absence of serosal HCO_3^- and CO_2 remains high in all species tested and leads to the prediction of high CO_2 production by the intestinal epithelium, which was confirmed by findings of intestinal tissue mass-specific metabolic rates being threefold higher than corresponding whole animal rates (1815). Furthermore, conversion of CO_2 to HCO_3^- is highly efficient as the majority of metabolic CO_2 is converted to HCO_3^- and secreted across the apical membrane (1815).

The high CO_2 hydration rates in the intestinal epithelium generate protons that must be secreted from the cells to maintain intracellular pH and continued CO_2 hydration. Secretion of protons occurs mainly across the basolateral membrane as the tissue exhibits high net base secretion rates and basolateral H^+-secretion has been measured directly. Inhibition of basolateral H^+ extrusion by removal of serosal Na^+ and application of ouabain (656) implicates basolateral NHE1 that is present in seawater-acclimated rainbow trout (595) and illustrates that Na^+/K^+-ATPase indirectly drives active transport of HCO_3^- (and Cl^-) across the apical membrane (656).

In addition to NHE1, the vacuolar H^+-ATPase is present in the basolateral as well as the apical membrane (658) and likely contributes to H^+-secretion across the basolateral membrane, although this remains to be demonstrated. In contrast, acid secretion across the apical membrane by the vacuolar H^+ pump has been demonstrated for at least two species (657, 662) and appears to be elevated in fish exposed to elevated salinity and most pronounced in the distal segments of the intestine (675). Apical H^+ secretion may seem counterintuitive in a net base secreting epithelium but likely serves osmoregulatory purposes. One consequence of apical H^+ secretion is greater potential for cytosolic HCO_3^- accumulation, in particular in the apical region rich in CAs and thus more favorable HCO_3^- gradients for continued anion exchange. In addition, apical H^+ pump activity will act to further polarize the apical membrane, which will also act to enhance anion exchange by the electrogenic SLC26a6. Thus, the H^+ pump contributes to intestinal Cl^- uptake and thereby solute-coupled water absorption. Finally, secretion of acid into the intestinal lumen will act to titrate and thus reduce the concentration of HCO_3^- in luminal fluids and thereby the osmotic pressure. Such reduced luminal osmotic pressure will further assist water absorption. It is important to appreciate that apical H^+ secretion does not prevent or reduce $CaCO_3$ formation in the lumen because resulting HCO_3^- concentrations are far in excess of luminal Ca^{2+} concentrations (see [652, 654, 662] for detailed discussions).

Kidney

Regardless of whether glomeruli are present, marine teleosts produce low urine flow rates of 0.03-0.9 ml/kg/h (115, 542, 749, 1203, 1204, 1206, 1208, 1713). Fish are incapable of producing concentrated urine, and while freshwater fish urine is dilute, urine of marine teleosts is isoosmotic to the extracellular fluids, with Mg^{2+}, SO_4^{2-} and Cl^- being the major electrolytes (115, 193, 747, 1204, 1206, 1663) (Table 6).

Tubule anatomy and function

Nephrons of the aglomerular kidney, which have been reported for ~ 30 teleost species, lack a renal corpuscle (115, 123). However, the majority of marine teleosts possess a glomerular nephron with a renal corpuscle containing the glomerulus. Common for marine teleost fish with glomerular and aglomerular kidneys is that almost all lack a distal nephron (384), which is responsible for Na^+ and Cl^- reabsorption in freshwater fish and other vertebrates (749). The tubules consist of two or three proximal segments that connect directly to the collecting duct via the connecting tubule (115, 123). The fact that loss of glomeruli has occurred several times during evolution and that urine composition from marine glomerular and aglomerular teleosts is near identical, illustrates that the renal contribution to marine teleost osmoregulation, regardless of the presence or absence of glomeruli is dominated by tubular secretion (115, 116, 118, 121, 123). Nevertheless, species with glomerular kidneys display filtration rates of ~0.5 ml/kg/h (see above).

Renal tubule secretion Na^+ and Cl^- are the dominants electrolytes in fluids secreted by isolated tubules from glomerular as well as aglomerular species. However, Mg^{2+} and SO_4^{2-} concentrations in tubular secretions are elevated far above plasma concentrations and thus may also contribute to fluid secretion (120, 309, 310). Tubular secretion of Na^+ and Cl^- is driven by Na^+/K^+-ATPase and presumably occurs much like it does across the gill (Fig. 18), with basolateral entry via NKCC1, paracellular movement of Na^+ and apical Cl^- secretion via cAMP-stimulated anion channels yet to be identified (120, 311). Although Mg^{2+} concentrations of 20 mM in tubular secretions (116, 121, 313, 1522) are lower than the ~140 mM observed in bladder urine (80, 596, 749, 1224), tubular Mg^{2+} secretion must be the product of active transport. Entry of Mg^{2+} across the basolateral membrane is thought to occur via Mg^{2+} channels with active transport across the apical membrane via Mg^{2+}:Na^+ exchange or Mg^{2+}:H^+ exchange driven by Na^+/K^+-ATPase and the H^+-pump, respectively (115, 1295, 1527). Like Mg^{2+}, SO_4^{2-} is also subject to tubular secretion, although bladder urine concentrations (596, 1203) by far exceed those observed in tubular secretions (10 mM) (116, 313). Regardless of glomeruli, active tubular SO_4^{2-} secretion contributes the most to renal excretion (124, 426, 1524) and is facilitated by basolateral and apical anion exchange as well as cytosolic carbonic anhydrase (1414, 1525, 1526). Basolateral SO_4^{2-}:OH^- exchange mediates SO_4^{2-} import while SO_4^{2-}/HCO_3^- or SO_4^{2-}/Cl^- exchange takes place across the apical membrane (1416). Most recently, the nature of the transporter responsible for apical secretion was identified to be an anion exchanger of the SLC26 family, an electrogenic SLC26a6 isoform (889), identical to the transporter responsible for Cl^-/HCO_3^- exchange in the apical membrane of the marine teleost intestinal epithelium (see above).

Renal tubular and urinary bladder absorption

The discrepancy between Mg^{2+} and SO_4^{2-} concentrations in tubular secretions and urinary bladder urine, discussed above, is the product of fluid absorption driven by Na^+ and Cl^- reabsorption in the distal proximal tubules and the urinary bladder, which results in low rates of urine flow and highly concentrated $MgSO_4$ (1167). Average glomerular fluid secretion rates are comparable to urine flow rates in marine teleosts, but fluid secretion rates may exceed glomerular filtration by as much as three- to four times (115). Thus, tubular fluid absorption rates are significant. The Na^+/K^+-ATPase creates favorable gradients for tubular Na^+-glucose and Na^+-amino acid cotransport, as well as Na^+/H^+ exchange coupled with apical Cl^-/HCO_3^- exchange (193, 384). The urinary bladder contributes to Na^+, Cl^- and water absorption and is responsible for absorption of as much as 60% of the urine *in vivo* (806). In contrast to the proximal tubule, Na^+ and Cl^- absorption in the urinary bladder is coupled and occurs via Na^+:Cl^- cotransporters resulting in absorption of isoosmotic fluid (577, 1096, 1521, 1523). Urotensin I and II secreted from the caudal neurosecretory urophysis may control transport by the urinary bladder epithelium (112).

Amphibia

The Amphibia were the first vertebrates that made the transition to land still depending, however, on the aquatic environment for reproduction. All modern amphibians belong to the subclass Lissamphibia that includes three orders: Anura (frogs and toads), Caudata/Urodela (newts and salamanders) and Gymnophiona (apodans or caecilians). Anuran species amount to 88% of a total of 6,771 recognized species within the subclass (561). The section on amphibian osmoregulation provides new perspectives for the combined role in ion transport by principal and MR cells of the epidermis and the subepidermal mucous glands as adaptations for aquatic and terrestrial life. With the kidneys responding fast to change in the availability of environmental water, the urinary bladder being a water storage organ, and anatomical, physiological and behavioral specializations for cutaneous drinking, amphibians are well adapted for prolonged stay on dry land. The putative selective pressure driving this evolution is an expanded niche exploration, and except for polar and high-altitude regions, modern amphibians are found in all climatic zones, and species from all three orders occupy a range of habitats from purely aquatic to purely terrestrial (456, 1756). While the natural history is well known for a large number of amphibian species, the physiology has been studied for a relatively small number belonging predominantly to the anurans. Aspects of amphibian osmoregulation are reviewed in (759, 764, 860, 1792, 1881).

Extracellular and Intracellular Ion Concentrations

In all three orders of amphibians the extracellular osmolality and the concentrations of Na^+ and Cl^- are lower than those of other vertebrates (see Table 7). The much larger concentrations, above 500 mosmol/kg H_2O, stem from burrowing desert species and species adapted to survive or live in coastal waters and estuaries (630, 894, 896, 1193). The extracellular K^+ concentration spans a remarkably wide range from less than 3 mM in *B. marinus* to 9 mM in *R. cancrivora* and *Ambystoma* with the lower values being characteristic of most studies. The high extracellular K^+ may reflect loss of K^+ from skeletal muscles because exercise (and anoxia) elevates plasma K^+ (1933). Among anurans, the concentrations of Na^+ and Cl^- vary between species and laboratory conditions. Amphibian plasma pH is between 7.8 and 8.1. The HCO_3^-/pCO_2 system is the major extracellular buffer and with pulmonary gas exchange and an arterial pCO_2 of ~1.6 kPa, the associated HCO_3^- is about 22 mM (161). In freshwater with cutaneous gas exchange or gill respiration the arterial pCO_2 is in the range of 0.4-0.7 kPa, resulting in plasma HCO_3^- of 7-10 mM at physiological extracellular

Table 7 Osmolality (π, mosmol/kg H_2O) and Concentration of Diffusible Ions and Urea (mmol/l) of Blood Plasma or Lymph as Indicated of Some Amphibians (mean ± sem or Range, Unless Otherwise Indicated)

	Blood plasma or lymph						
Species	π	Na^+	K^+	Cl^-	HCO_3^-	Urea	Remarks
				Anura			
Rana ridibunda (892)	247±12	115±5	6±1	83±6		11±2	In tap water
Rana pipiens (1261)	193±8	112±3		68±3			Semiaquatic, kept in about 0.2 mM NaCl
Rana cancrivora (630)	290±10	125±17	9±1	98±10		40±1	In fresh water
Rana catesbeiana (1769)	214.6±7.5	100.2±5.1	2.5±0.6	58.5±6.5	27.1		mean±sd 20°C
Bufo boreas (1261)	235±15	109±3		77±5			Terrestrial, collected in the field
Bufo viridis (1679)	275±5	120±6	4.0±0.4	87±2		17±2	In tap water
Bufo bufo (1308)		119±2	3.0±0.25	79±2			In tap water, lymph samples, mean±sd
Bufo bufo (847)		105.5±1.0		85.4±2.6			Terrestrial habitat free access to tap water and mealworms
							Lymph samples
Bufo marinus (1264, 1769)	234±10	107.9±6.8	2.4±0.4	76.1±4.9	24.1	—	mean±sd 20°C
Bufo marinus (960)	241.2±3.4	99.8±2.6	4.6±0.1	72.6±3.8		19.6±3.2	
Bufo marinus (586)	209.4±3.9	126.7±2.4	3.64±0.20			—	Distilled water adapted
Bufo marinus (586)	300.2±3.8	185.5±4.7	3.59±0.28			—	Saline adapted 0.9% NaCl
Hyla regilla (1261)	218±10	110±3		78±5			Terrestrial, collected in the field
Ascaphus truei (1261)	172±5	106±3		81±3			High altitude streams, collected in the field
Scaphiopus couchi (1193)	225-390 253-346	141-210 120-174	3.6-14.4 3.6-5.7	84-152 92-125		37-173 18-51	Collected from temporary pools
	590 630	204 228	5.4 5.4	159 165		228 286	2 toads emerging from ground borrows
Rana cancrivora (630)	290±10	125±17	9±1	98±10		40±1	In fresh water
	830±50	252±12	14±0.5	227±9		350±1	In 80% saltwater
Bufo viridis (894)	296±17					22.2±12.3	In tapwater
	597±11					158±50	In 580 mosmol/kg NaCl (mean±sd)
(896)	392±15	141±6		110±10		32±13	Access to tap water
	752±27	162±8		162±10		272±16	Burrowing

Table 7 (*Continued*)

Species	Blood plasma or lymph						
	π	Na^+	K^+	Cl^-	HCO_3^-	Urea	Remarks
Caudata							
Ambystoma gracile larvae (1764)	191±2	94.4±2.4	9.5±0.6	75.4±1.7			In tap water at 15 °C for ≥ 1 week unfed for 2 weeks
Ambystoma tigrinum (1670)		106	9	98			
Ambystoma tigrinum larvae (730)		107.9±1.3	5.2±0.3				In tap water at 5 °C, unfed for ≤2 months
Necturus maculosus (730)		93	2.5	82			
Batrachoseps attenuates (854)	339±8.9	110±7.0				48±8.2	Tap water at 15 °C unfed < 1 week
Gymnophiona							
Typlonectes compressicauda (1765)	196.3±3.2	111.4±5.3	5.4±0.5	69.7±4.8			In tap water at 25 °C, fed *Tubifex* worms
Ichthyophis kohtaoensis (1765)	220±5	101.3±9.0	3.2±0.3	87.5±1.9			Housed in damp potting soil at room temperature, access to live crickets
Ichthyophis kohtaoensis (1765)	210.6±4.8	111.9±3.6	2.7±0.3	79.5±2.7			In tap water

pH of about 7.8 (230). The distribution of diffusible ions between extracellular and intracellular fluids conforms to the usual picture: cellular Na^+ is significantly below and cellular K^+ above thermodynamic equilibrium (Table 8). In principal cells of the epidermis and acinar cells of subepidermal exocrine glands, Cl^- is above thermodynamic equilibrium, whereas in striated muscle cells and in MR cells under terrestrial conditions Cl^- is passively distributed. In freshwater the electrochemical potential of Cl^- would be above that of the environment because of active uptake across the apical membrane of MR cells.

Body Water Volumes

Amphibians contain relatively more water than do other vertebrates (Table 9). The total body water constitutes 70%-80% of the body mass of which the intracellular fluid amounts to 70%-80% of the total water. The extracellular fluid is divided between interstitial fluid, blood plasma and the lymphatic system. It is an intriguing aspect of anuran osmoregulation that they are tolerant to significant perturbations of body water volume. Among the species studied, the extracellular water volume ranges from 24% to 48% (Table 9) associated with a highly variable lymph volume, with little variation in plasma volume (hematocrit), and thus unimpaired circulation (753, 757, 760, 781, 868, 1193). This owes to a fast exchange of fluid between blood plasma, interstitial, and lymph volumes (79, 332, 333, 755, 758, 1220) governed by a high whole-body systemic transcapillary filtration coefficient (692). The tolerance to desiccation of anurans is to some degree correlated with their terrestriality (755, 756, 1829), with the limit set by the volume loss impairing the circulatory system caused by an increased hematocrit (1670). The limit may be surprisingly high as indicated in a study of the aquatic *Xenopus laevis*; at an upper-limited/non-tolerated water loss of 33.8±1.0% of the body mass, the extracellular osmolality was 523±14 mosmol/kg with a hematocrit of 65.5±2.4% (754).

Epidermal Epithelium of the Skin

The skin of amphibians is a major site for ion and water exchange. Extensive studies have been performed on adult stages, mostly of anurans as discussed below, but larval stages also have been investigated (432, 911, 940, 941, 943). In larval stages, gills constitute an additional site for ion and water

Table 8 Intracellular Concentrations of Small Diffusible Ions and Membrane Potentials of Striated Muscle Fibre and Anuran Epithelial Cells with Osmoregulatory Function

	Na^+	K^+	Cl^-	HCO_3^-	V_m
Tissue	mmol/l				mV
Striated muscle fibre	10.4[1]	124[1]	1.5[1]	12.4[1]	−95[2]
Acinus cells of subepidermal mucous gland	11.5[3]	155[3]	55[3]	—	−69.5±0.7[4]
Principal cell compartment of epidermis, bilateral Ringer's solution	9.9[5]	159[5]	49[5], 50[6]	—	−108±2[7]
Mitochondria-rich cells of epidermis, bilateral Ringer's solution	30.0[8]	150[8]	47.9[8] 38.4±3.9[9] 14[10], 25[11], 19.8±1.7[12]	—	−33.2±2.5[9]
Principal (granular) cells, toad urinary bladder	19.5[13]	163[13]	57.5[13]	—	−94[14] −57.2±2.3[15]
Mitochondria-rich cells, toad urinary bladder	11.2±2.5[16]	114.4±3.7[16]	—	—	–

[1](336). [2](1231). [3]X-ray microanalysis (774). [4]Basolateral membrane potential of resting gland cells (1733). [5]X-ray microanalysis (1545), here and in the following corrected for gram dry mass per 100 g wet mass according to the information given in the respective papers. [6](1126). [7]Basolateral membrane potential (1274). [8]X-ray microanalysis (1542). [9]GHK fit of single-channel i_{Cl}/V relationships in cell attached patch on cells in free suspension, i.e., short circuited cells (1732).[10]X-ray microanalysis (1546). [11]X-ray microanalysis (1543). [12]From estimated intracellular Cl^- space of MR cells exposed to bilateral NaCl Ringer's solution (1007). [13]X-ray microanalysis (1544). [14]Basolateral membrane impalement of short-circuited cells (1284).[15]Basolateral membrane impalement of short-circuited cells (439).[16]X-ray microanalysis in mmol/kg wet mass, i.e., here the concentration is not corrected for dry mass of analyzed sections (1544).

exchange (64, 98, 1039-1043), but the quantitative role in maintaining larval ion balance is not yet fully established. Amphibians do not drink orally; the colon epithelium, however, expresses luminal Na^+ channels that are regulated like the apical Na^+ channel of the skin (253, 326, 399, 965-967, 1038). Under terrestrial laboratory conditions, whole-body ion balance of Na^+ and Cl^- in *B. boreas* and *R. pipiens* depends on ingested food (759). Generally, for aquatic environments quantitative studies of the relative significance of dietary and cutaneous ion intakes are lacking.

Pioneered by Hans Ussing, in several years following World War II, frog skin served as one of the preferred experimental preparations in the study of active ion transport and functional organization of transporting epithelia (991, 992). The first model of a NaCl-absorbing epithelium was developed for frog skin (956), which influenced studies of ion transport by epithelia with osmoregulatory functions generally (508, 1388). The micrograph of Fig. 20 shows that anuran skin comprises a heterocellular, multilayered absorbing epithelium with principal cells and MR cells (219, 220, 484, 520, 1742, 1980), and subepidermal glands (495, 1230, 1322, 1323). Numerous studies on amphibian skin have extended the original model, which today includes observations on a large number of species considering additional ion exchange pathways, water channels, regulatory mechanisms and functions of the subepidermal glands. Figure 21 shows updated models of the functional organization of anuran epidermis. Because of the transitional position of adult amphibians, the models of Fig. 21 indicate adaptations both for the life in freshwater and on land as reflected by the apical aquaporin water channels of principal cells and pathways for both active and passive Cl^- uptake of MR cells. Further to this, regulatory mechanisms at cell level have evolved for meeting a lifestyle that comprises both aquatic and terrestrial adaptations. Each of the three transporting units shown in Fig. 21—(i) principal cells, (ii) MR cells and (iii) subepidermal glands—is configured for specific functions related to amphibian osmotic and ionic regulation. They are dealt with in separate paragraphs below, which also discuss the functional interplay between the two epidermal cell types as well as the coupling of epidermal and subepidermal functions in osmoregulation.

Principal cell compartment

The multilayered principal cell compartment that amounts to 90%-95% of the total epidermis is specialized for active uptake of Na^+ (1894). The cells of all layers constitute a functional syncytium enclosed by the apical plasma membrane of the outermost living cell layer and by the plasma membranes lining the labyrinth of intercellular spaces (519, 520, 1273, 1545, 1656, 1718, 1893). Gap junctions are included in this

Table 9 Amphibian Body Fluid Volumes Expressed as Percent of Total Body Mass (Hematocrit: % Blood Cells). Where Indicated, Total Body Water (TBW) is Calculated Relative to the Mass with the Urinary Bladder Emptied, Which Defines a "Standard Body Mass" (1671)

Species	TBW	Extracellular vol. Marker	Plasma vol. Marker	Hematocrit % cells	Remarks
Rana pipiens	78.9[1]	23.6-29.8[2]	5.6-9.3[2]		
		Thiocyanate	T-1824		
Rana catesbeiana	79.0±0.5[11]	29.8-47.9[2]	7.2-8.8[2]	30±5.3[3]	Ref. (1827) unfed in tab water for ≥ 2 weeks
		Thiocyanate	3.7±0.3[11]	22-34[11]	
		21.7±1.0[11]	T-1824		
		Sucrose			
Hyla cinerea	80.1[1]				
Scaphiopus hammondii	80.0[1]				
Scaphiopus couchi	69.8-81.6[4]				
Aneides flavipunctatus	71-78[5]				
Bufo bufo	81.3±1.0[6]*	32.0±1.6 [7] ^{14}C-inulin			*TBW of lean body mass Empty urinary bladder
Bufo marinus	74.1±0.7[11]	24.7±1.5[11] Sucrose	4.7±0.5[11] T-1824	37±1.7[3] 26.4±2.2[9] 27-40[11]	Ref. (1827) unfed in tab water for ≥ 2 weeks
Bufo viridis	72.7±0.9[8]	35[8] ^{14}C-inulin	6.3[8] Evans blue		Free access to tap water Empty urinary bladder
Necturus maculosus	81.1±0.5[11]	24.1±0.7[11] Sucrose	4.7±0.2[11]	28-38[11]	Ref. (1827) unfed in tab water for ≥ 2 weeks
Cryptobranchus alleganiensis	79.1±0.5[11]	22.0±1.0[11] Sucrose	3.5±0.16[11] T-1824	36-45[11]	Ref. (1827) unfed in tab water for ≥ 2 weeks
Amphiuma means	78.8±0.3[11]	21.8±0.8[11] Sucrose	3.4±0.2[11] T-1824	18-37[11]	Ref. (1827) unfed in tab water for ≥ 2 weeks
Ambystoma gracile larvae		32[10] ^{14}C-inulin			
Dicamptodon ensatus larvae		23[10] ^{14}C-inulin			

[1](1829). [2](1487). [3](758). [4](1193). [5](1513). [6](1308). [7](870). [8](781). [9](961). [10](1764). [11](1827).

compartment mediating the diffusion of small ions between cells. Tight junctions between the outermost living cells constitute high-resistance seals (422, 497, 519) of permeability to Na^+ that is slightly above the permeability to Cl^- (995, 1261).

Following publication in 1958 of the frog skin model (956), it was discussed that the electrical potential step at the apical membrane is positive. This would be expected if the Na^+ conductance of the apical plasma membrane is much larger than that of K^+ of the basolateral plasma membrane. Subsequent well-controlled cell impalements with microelectrodes showed that the cell is electrically negative with respect to the outside bath, indicating that the K^+ conductance of the basolateral plasma membrane is much larger than the apical Na^+ conductance (709, 729, 1274). This implies that the apical plasma membrane potential constitutes an additional and major driving force for Na^+ uptake across the outward facing membrane.

Apical Na^+ channels: ENaC The apical Na^+ channel of the outward-facing membrane is blocked by a concentration of amiloride in the low micro-molar range (1067, 1897). The channel's molecular phenotype as exemplified by its selectivity, rectification, single channel conductance and amiloride sensitivity indicated that it belongs to the large family of epithelial sodium ion channels denoted ENaCs. This has been verified in a more recent study of cloning and functional characterization of the amiloride inhibitable sodium channel of the skin of adult *R. catesbeiana* (849). The uptake of Na^+ is stimulated by the amphibian neurohypophyseal hormone, arginine vasotocin (AVT) (93) and by aldosterone (1276), which also stimulates the basolateral conductance (1279). It

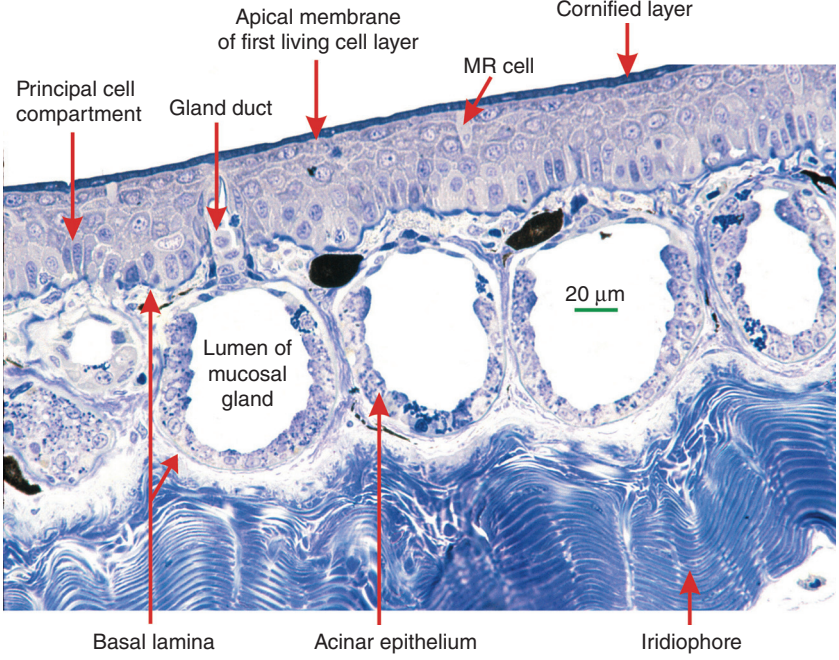

Figure 20 Anatomy of anuran skin (*Rana esculenta*) with two ion and water transporting units. The multilayered, heterocellular epidermis is an absorbing epithelium of principal cells and MR cells. The acinar epithelium of the subepidermal mucous glands is secretory. Courtesy Dr. Åse Jespersen (993).

is a halmark of the Na^+ channel of the anuran skin, that the open probability is under fast control by the external Na^+ concentration via an external Na^+-specific binding site (562, 1899). The Michaelis-Menten-like saturation kinetics of the unidirectional Na^+ influx (128, 498, 938) is due to this mechanism and not to saturating carrier transport with which it is easily mistaken. It is conceivable that this mechanism prevents swelling of the epithelial cells when external [Na^+] suddenly changes from the low concentration of freshwater to the much higher concentration of Ringer's solution of signifiance when the animal is on land and covered by a near-isotonic surface fluid produced by the subepidermal glands (see below). Independent of this regulation of the apical sodium permeability (P^a_{Na}), Harvey and Kernan provided evidence for a negative feedback control of P^a_{Na} by the *intracellular* sodium ion activity (710).

Apical K^+ channels The apical K^+ channels shown in Fig. 21A constitute a route for cutaneous elimination of K^+ in animals experiencing a body load of potassium (552, 1278, 1310, 1900). This pathway, exhibiting single filing (504), is normally downregulated, rendering the apical membrane of principal cells Na^+ selective as suggested in the original frog skin model.

Apical Cl^- channels are missing The apical membrane of principal cells is tight to Cl^- even after activation of the transepithelial passive Cl^- permeability (1271, 2001, 2002), leaving the Cl^- uptake to other pathways.

Localization and functions of the 'sodium pump' The active uptake of Na^+ is fueled by the ouabain-sensitive, rheogenic $3Na^+/2K^+$ P-ATPase (344, 503, 709, 1275, 1312), which is expressed exclusively in the plasma membranes lining the lateral intercellular spaces (518, 1227, 1229). The pump maintains the intracellular Na^+ concentration below and the intracellular K^+ concentration above thermodynamic equilibrium (580, 709, 1008, 1277, 1313, 1545, 1774) (see Table 8). The intracellular [Cl^-] is also above thermodynamic equilibrium (129, 616, 709, 1277, 1545, 2001), which seems to be maintained by a basolateral Na^+-$2Cl^-$-K^+ cotransporter in parallel with a low-conductance passive basolateral Cl^- permeability (469, 470, 1890). These mechanisms play a role in cell water volume regulation in frog skin principal cells (528, 1890, 1891) and in body cells more generally (287, 783).

K^+ channels at the basolateral membranes The relatively high K^+ permeability of the basolateral plasma membrane results in an electrical conductance that is several times larger than that of the apical plasma membrane, and an intracellular electrical potential that is negative with respect to both the outside bath and the interstitial fluid (709, 729, 1274). The membrane's ion conductance has been characterized by noise analysis and ion channel inhibitors (765, 1898). Intracellular pH, conceivably controlled by basolateral Na^+/H^+—and Cl^-/HCO_3^- exchange mechanisms (Fig. 21A), regulates the open/close kinetics of the apical Na^+—and the basolateral

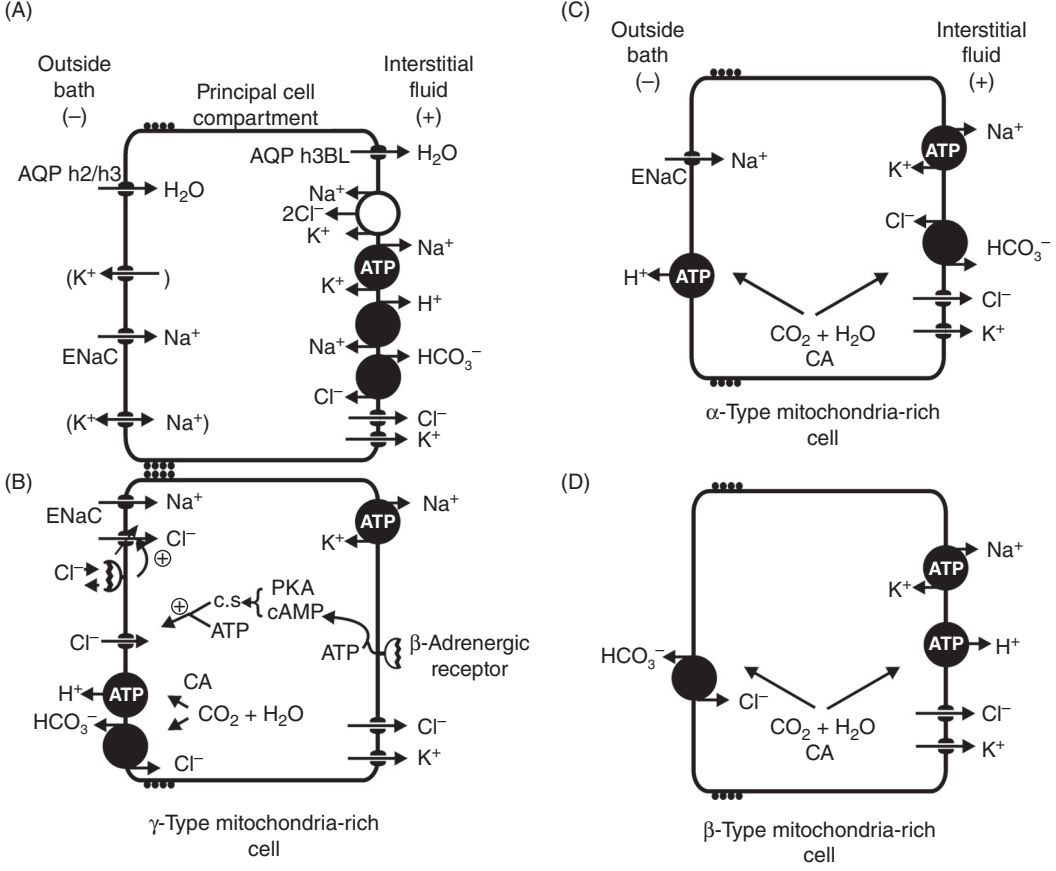

Figure 21 Models of the functional organization of the heterocellular anuran skin epithelium. Experimental approaches and methods are detailed in the text. (A) The large Na^+-transporting syncytial compartment. Cl^- channels are absent in the apical membrane, which contains Na^+ channels (ENaC) and K^+ channels. *Notably, the Na^+/K^+ pumps are located exclusively in the plasma membranes lining the lateral intercellular spaces*. (B) The γ-type MR cell specialized for active and passive Cl^- uptake as has been studied in frogs in freshwater and on land covered by saline of regulated composition, respectively. Shown at the apical plasma membrane are voltage- and concentration-gated Cl^- channel with its regulatory external Cl^- binding site, and the hormone-gated CFTR like Cl^- channel. PKA: protein kinase A; c.s.: catalytic subunit; CA: carbonic anhydrase. As discussed in the text, a number of observations on acid-base transport and active Cl^- uptake are not compatible with the hypothetical γ-type MR cell. They are, however, reconciled with two other types of MR cells (α and β), originally discovered in turtle urinary bladder. (C) Accepted model of an acid-secreting α-type MR cell. (D) Accepted model of a base-secreting β-type MR cell with associated non-rheogenic active Cl^- uptake. Modified from (993).

K^+ channels (706, 711, 1886), supposed to regulate the intracellular cation pools when the animal is facing environmental conditions of varying external Na^+ concentrations. Another function of basolateral K^+ channels is to maintain a sufficient electrical driving force for the passive Na^+ uptake through the apical membrane's Na^+ channels (1272), even at an external Na^+ activity as low as 100 μM, that is, well below that of many freshwater pools (709). Thus, the relatively high and regulated basolateral K^+ permeability is of significance both for maintaining intracellular ion pools and cell volume, and for maintaining whole body extracellular Na^+ balance. It is likely that different types of K^+ channels control these functions. However, they have not yet been identified genetically, which contrasts the fairly detailed description of their biophysical properties that include single-filing (345), anomalous rectification (1885), and ATP dependency (1887).

Water channels Another important function of the principal cell compartment is to regulate transcutaneous uptake of water (761, 1126, 1738). This function is mediated by insertion of aquaporin isoforms, AQP-h2 and AQP-h3, into the apical plasma membrane, which has been studied by AVT-stimulation via V2 receptors (14, 712, 1806), isoproterenol stimulation via β-adrenergic receptors, and hydrin-1, and -2 stimulation via V2 receptors (1364). Water passes the basolateral plasma membrane through constitutively expressed aquaporins characterized as AQP-h3BL (712, 1363, 1791).

MR cells

Mitochondria-rich or mitochondrion-rich (MR) cells display two types of plasma membrane ion pumps: the P-type Na^+/K^+ pump and the V-type H^+ pump. Anuran skin has the capacity

to acidify the external bath by a H^+ pump (482, 492, 1119), which by immunocytochemical labeling was identified as a V-type H^+ pump in the apical plasma membrane of MR cells in the skin of the semiaquatic frog, *R. esculenta* (483, 945). A similar pump was subsequently identified in the skin of the terrestrial *Bufo bufo* (847). The pump that seems to correlate with the previously identified rod-shaped intramembranous particles of MR cells (207) translocates one positive charge outwardly for each proton excreted (708, 846). There is evidence that proton pumps are expressed in three types of MR cells of anuran skin that serve different functions. This issue is dealt with first. Subsequently, the function of the Na^+/K^+ pump of MR cells is discussed. The passive Cl^- transport is also mediated by this minority cell type, which is discussed toward the end of the paragraph on MR cells.

Role of the H^+ ATPase of MR cells in energizing Na^+ uptake in freshwater The electrical potential difference provides a major driving force for Na^+ uptake across the outward-facing membrane, which secures uptake of Na^+ also at reduced external Na^+ concentrations. Ehrenfeld, Garcia-Romeu and Harvey (482) reasoned that at very low external Na^+ concentrations the driving force for Na^+ transport across the apical membrane would reverse, because the membrane potential would no more overcome the chemical potential difference across this membrane. They hypothesized that *in vivo* the rheogenic H^+ pump by hyperpolarizing the apical membrane energizes the cellular uptake of Na^+ from diluted solutions (482, 708). Following their localization of the proton pump to MR cells, they modified the hypothesis by pointing out that a similar effect would be obtained at open circuit conditions by the external current loop carried by the H^+ flux through MR-cells and the Na^+ flux through principal cells (483, 707).

The H^+ ATPase of the γ-type MR cell energizes active transcellular Cl^- transport In the late 1930s, August Krogh discovered a powerful active Cl^- uptake mechanism in the skin of *R. esculenta* (973, 978) subsequently found in other species as well (24, 583, 871). It displays half-maximum saturation concentration in the range of 100 μM to 500 μM of external Cl^- (20, 24, 213, 581, 1261). Ehrenfeld and Garcia-Romeu (480) showed that Cl^- uptake at low concentrations is dependent on and tightly coupled to excretion of HCO_3^- (see Fig. 22).

The exchange mechanism is independent of the transepithelial potential difference (480, 848, 970), exhibits *cis* side interaction between $^{36}Cl^-$ and ordinary chloride (848) and is inhibited by carbonic anhydrase inhibitors (20, 213, 480, 583). The enzyme is expressed exclusively in MR cells (1578) like an apical band-3-related protein (421, 895). Taken together, these findings provide compelling evidence for an apical Cl^-/HCO_3^- exchange of MR cells governing Cl^- uptake in freshwater. Several studies have shown that the active Cl^- flux is rheogenic (111, 213, 1173, 2064). It was hypothesized, therefore, that the active uptake of Cl^- (and of Br^- and I^- as well) is energized by the proton pump of the γ-type MR cell shown in Fig. 21B, which is depicted with both the Cl^-/HCO_3^- exchanger and the V-type H^+ ATPase in the apical membrane (1008). Further support for this hypothesis comes from studies of toad skin (*B. bufo*) demonstrating that replacement of external Cl^- by a non-permeating anion reversibly acidified the external bath as predicted by the model of Fig. 21B (492, 846, 1008). In skins of salt-depleted anurans (*R. esculenta* and *B. bufo*) bathed with low NaCl on the outside, the H^+ efflux and the Cl^- influx were of similar magnitude. Both fluxes were reduced by about the same amount by the V-type H^+ pump inhibitor concanamycin A, confirming that proton pumps energize active uptake of Cl^- (848).

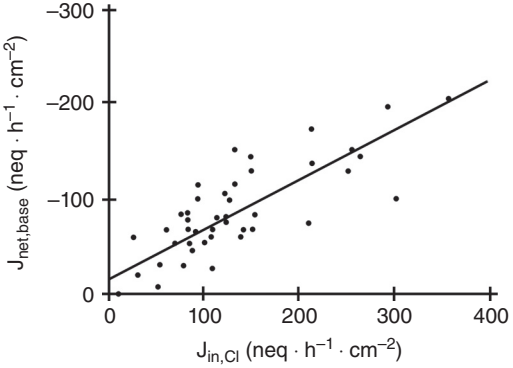

Figure 22 Relationship between Cl^- influx ($J_{in,Cl}$) and net base excretion ($J_{net,base}$) in short-circuited frog skin (*R. esculenta*) exposed to 2 mM Cl^- on the outside and SO_4^{2-} on the inside. Active Na^+ flux is blocked by amiloride. The regression line is drawn according to

$$J_{net,base} = -(0.50 \pm 0.06) \cdot J_{in,Cl} - (16.7 \pm 9.3),$$
$$r = 0.78 \pm 0.15, P < 0.001.$$

The slope of ~0.5 is compatible with the finding that the anion exchange mechanism also mediates Cl^- self-exchange. Adapted from ref. (480).

With both the H^+ pump ATPase and the Cl^-/HCO_3^- exchanger located at the apical membrane the functional organization of this anuran MR cell-type are clearly different from MR cells serving whole-body acid/base regulation. Thus, it has been denoted the γ-type MR cell. As discussed in detail below, the apical membrane of the γ-MR cell is also the site of chloride channels that are activated when the frog is on land and covered by a cutaneous surface fluid generated by submucosal glands.

Role of the H^+ ATPases of α- and β-type MR cells in eliminating acid and base loads The H^+ pump ATPase energizes cutaneous elimination of protons in acid-loaded frogs (*R. esculenta*), which display an increased density of cutaneous MR cells and increased number of H^+ pumps inserted into the apical plasma membrane by exocytosis in response to acid loading (707). Aldosterone stimulates the active proton secretion and a simultaneous uptake of Na^+ via

ENaC, which secures electroneutral transepithelial transport in open-circuit conditions. Because H^+ secretion into the outside bath was associated with base secretion into the inside bath (465), it is conceivable that this function is mediated by an α-type MR cell depicted in Fig. 21C, originally discovered in turtle urinary bladder (1759) and now also studied in epithelia of other lower vertebrates (for further discussion, see section above on Agnata and Pisces). This hypothesis conforms to the early observation that in frog skin (*R. ridibunda*), external acidification was unchanged if sulphate was replaced by chloride on the corneal side of the skin (493), confirming that a Cl^-/HCO_3^- exchange mechanism is expressed at the basolateral rather than apical plasma membrane. There is evidence that toad skin also contains α-MR cells. Firstly, double immunostaining showed expression of apical proton pumps and basolateral AE1/AE2 Cl^-/HCO_3^- exchangers in flask-shaped MR cells in the skin of *B. marinus* (206). Secondly, in 9 out of 42 preparations of *B. bufo*, acidification of the outside bath was independent of the outside anion. Throughout the year, the toads were kept in a simulated terrestrial habitat at room temperature with free access to a pool of tapwater and fed mealworms twice a week. Thus, it is worth noting that seven of the nine preparations were studied during a one-month midsummer period, which would indicate seasonal variation of the functions of toad skin MR cells (846).

MR cells with H^+ pumps in the basolateral membrane and Cl^-/HCO_3^- exchangers in the apical membrane are configured for active non-rheogenic uptake of Cl^- (conf. Fig. 21D). This so-called β-type MR cell serves base secretion in distal renal epithelia (1759, 1919). Base-loaded frogs excrete HCO_3^- via the skin (1903), and the classical study by Garcia-Romeu and coworkers indicated non-rheogenic active uptake of Cl^- in exchange for base in frogs selectively depleted of Cl^- (583). These observations would be in agreement with the hypothesis that anuran skin under some conditions contains β-type MR cells.

Putative function of active Na^+ transport by MR cells

MR cells display apical amiloride sensitive Na^+ channels and basolateral Na^+/K^+-pumps and are thus configured for transporting Na^+ actively into the interstitial fluid (707, 1007, 1542). With Ringer's solution on the outside, the flux amounts to a very small fraction of the active Na^+ flux via principal cells (1007, 1542). It is not clarified, however, whether the uptake of Na^+ by MR-cells is of physiological significance in highly diluted solutions. Regarding this question, it is interesting that the "saturation kinetics" of Na^+ uptake in anurans at $Na^+_{bath} < 3$ mM exhibits a $K_{1/2}$ of 200-300 μM (481, 647, 932, 939). Similar low $K_{1/2}$ values were observed in the caecilians, *Typhlonectes compressicauda* and *Ichtyophis kohtaoensis* (1765). The nature of the Na^+_{bath}-dependence of the Na^+ uptake in highly diluted solutions deserves further studies.

Passive Cl^- transport by the γ-type MR cell Experimental studies applying different methods have indicated that

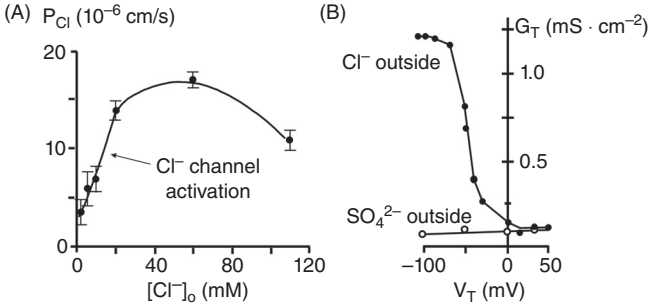

Figure 23 Studies by Larsen et al. of the dynamic apical Cl^- channels of MR cells of anuran skin. (A) Dependence of the permeability, P_{Cl}, on Cl^- concentration of outside bath at constant V_T ($\psi_o - \psi_i$) = -80 mV (inwardly directed electrical force on Cl^-); as Cl^-_o approaches freshwater concentrations, the channels close reversibly. Adapted from (699). (B) Dependence of the Cl^- conductance on V_T at constant $[Cl^-]_o$ = 110 mM. G_{Cl} is activated in the physiological range of transepithelial potentials—that is, Cl^- channels open when the driving force on Cl^- is in the inward direction ($V_T < 0$ mV) and close when the driving force is outwardly directed ($V_T > 0$ mV). Adapted from (995).

MR cells constitute the physiological pathway for passive transepithelial uptake of Cl^- in anuran skin (546, 901, 996, 1007, 1280, 1917, 2001). The passive Cl^- conductance (G_{Cl}) of MR cells in the skin of frogs and toads is deactivated in the absence of Cl^- on the outside (956) and reversibly activated at external Cl^- concentrations above 3-5 mM (24, 699, 940) (see Fig. 23A). This G_{Cl}, which is controlled by external Cl^- via an apical chloride-binding site as indicated in Fig. 21B, has poor anion selectivity. The preference for Cl^- above other anions has been ascribed to an external binding site with a high Cl^- specificity, which allows the channel to open at external Cl^-_o above ~5 mM (699, 971), see ref. (993) for detailed discussion. In the skin of toads, *B. bufo* (994, 995), *B. viridis* (897) and *B. marinus* (985, 1007), for $Cl^-_o > 5$ mM, G_{Cl} is also potential dependent in such a way that depolarization of the apical plasma membrane of MR cells activates G_{Cl} (994) in the Ussing chamber accomplished by hyperpolarization of the skin potential (995). This seems different from the skin of frogs (e.g., *R. esculenta*, *R. temporaria* and *R. pipiens*), which does not always show a similar V_T dependence of G_{Cl} unless prior stimulated by theophylline, cAMP or forskolin added to the serosal bath (972, 1583). The Cl^- conductance is slowly activated (30-180 s) following a hyperpolarizing step of V_T ($= \psi_{\text{outside bath}} - \psi_{\text{inside bath}}$), and deactivated at $V_T > 0$ mV (897, 900, 903, 985, 995, 1003, 1583, 2000, 2001). As a result, the steady state G_{Cl} depicts an inverted S-shaped function of V_T (see Fig. 23B).

In voltage clamp experiments, the Cl^- uptake across the isolated skin epithelium is associated with volume expansion of MR cells (546, 1007) of a time course similar to that of the transepithelial Cl^- current activation (1007). At fully activated G_{Cl}, the Cl^- current through single MR cells is quite impressive, ranging from about −1 to about −15 nA (546, 996, 1280). Noise analysis of whole cell Cl^- currents indicated a large single channel conductance of about 250 pS

(994), which was indicated also in single-channel recordings of isolated MR cells (1732). The passive Cl^- permeability of MR cells is controlled also via β-adrenergic receptors at the basolateral membrane and stimulated by forskolin and membrane permeable cAMP analogues (405, 898, 902, 972, 1282, 1283, 1582, 2002). In toad skin, this eliminated the time and potential dependence of G_{Cl} within the physiological range of transepithelial potentials (1582, 2002), associated with the activation of a small (8-10 pS) membrane-potential-independent CFTR-like Cl^- channel of the apical membrane (1732). As mentioned above, in studies of the skin of *R. esculenta, R. temporaria* and *R. pipiens*, similar treatments were reported often to be a prerequisite for G_{Cl} being activated by hyperpolarizing V_T (972, 1583). The γ-MR-cell shown in Fig. 21B depicts these functions as governed by two different apical Cl^- channels in agreement with whole cell noise analysis (994) and single channel recordings (1732). As an alternative and still possible interpretation, Katz, Nagel, and Rozman hypothesized that phosphorylation and potential stimulation target a common apical Cl^- pathway (1281, 1583). In line with this hypothesis, it has been discussed that large-conductance Cl^- channels are composed of small Cl^- channels of coordinated activity (2000). This interpretation would be compatible with observed single-channel i_{Cl}/V-relationships of the big Cl^- channel that did not exhibit a common GHK-permeability, but a range from $0.3{\cdot}10^{-12}$ cm^3/s to $1.18{\cdot}10^{-12}$ cm^3/s (1732).

Dynamic coupling of active Na^+ uptake by principal cells and passive Cl^- uptake by MR cells

At open-circuit conditions where V_T governs passive Cl^- uptake via MR cells, the rheogenic active flux of Na^+ and the simultaneous electrodiffusion of Cl^- constitute a current loop across the epithelium. It follows that stimulation of the active Na^+ flux through principal cells—for example, by increasing the number of open ENaC channels—will increase the current through MR cells, leading to an ohmic depolarization of their apical plasma membrane that in turn activates Cl^- channels. The range of V_T in which the Cl^- permeability is taken from deactivated to fully activated state (Fig. 23B) is similar to the voltage range generated by the active Na^+ flux when the apical Na^+ permeability of principal cells is passing from non-activated to fully activated state. Quantitative analysis showed that this type of regulation results in tight coupling of the active Na^+ flux by principal cells and the passive Cl^- flux through MR cells (993, 1004).

Passive and active Cl^- transport—the frog on land and in freshwater

The concentration and voltage dependence of the passive Cl^- permeability discussed above seem to be an adaptation of amphibians to a life alternating between freshwater and land (993). On land, where the skin is covered by a thin layer of saline produced by subepidermal exocrine glands (see below), reuptake of NaCl (and water) supposedly constitutes a major function of the epidermal cells. In freshwater, closure of the epidermal Cl^- channels prevents cutaneous loss of Cl^- to the environment so that not only Na^+ but also Cl^- is taken up by active transport. The interrelationship between these functions has been studied experimentally by flux-ratio analysis of unidirectional Cl^- fluxes (e.g., 990). For Cl^- at 20 °C, Eq. 12d is

$$\log_{10}\frac{J_{Cl}^{in}}{J_{Cl}^{out}} = \frac{z_{Cl}(V_T - E_{Cl})}{58.0} \qquad (14)$$

Figure 24 shows the relationship between the measured flux ratio and the external driving force, $z_{Cl}{\cdot}(V_T - E_{Cl})$, which is positive in the inward direction. The theoretical flux ratio is indicated by the straight line of slope, $10^{-3}{\cdot}F{\cdot}\log_{10}e/(R{\cdot}T) = 1/58\ mV^{-1}$. For an inwardly directed driving force, Cl^- channels are open, allowing for a passive flow of Cl^- from bath to interstitial fluid (see the right-hand side of the diagram). For $V_T > 0$ mV, the Cl^- channels close, revealing the active component of the transepithelial Cl^- flux (open symbols). This component is seen also when external Cl^- is reduced to freshwater concentrations (conf. the closed symbol of Fig. 24). With the apical Cl^- channels closed, in freshwater,

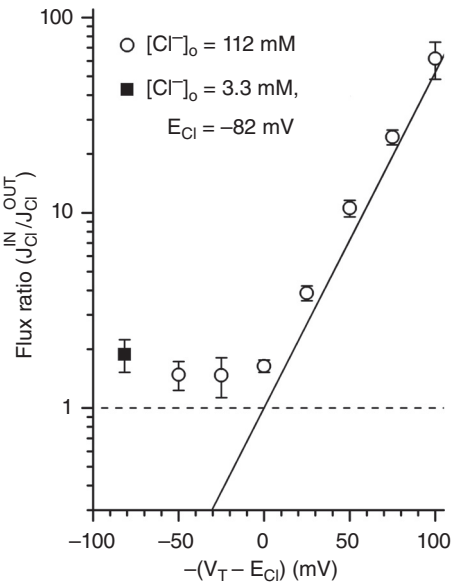

Figure 24 Flux-ratio analysis of Cl^- transport across the isolated skin of B. bufo. The straight line was calculated by Eq. 14 in the text. The graph shows that the experimental flux ratio follows the theoretical line for electrodiffusion if the driving force, $z_{Cl}{\cdot}(V_T - E_{Cl})$, is in the inward direction at elevated external Cl^-, where the apical Cl^- channels of the γ-MR cells are activated (right-hand side). If $z_{Cl}{\cdot}(V_T - E_{Cl})$ is in the outward direction (left hand side), the apical Cl^- channels are closed while the active Cl^- fluxes, fueled by the apical H^+ V-ATPase, and the Cl^-:Cl^- exchange diffusion fluxes become the dominating modes of Cl^- transport. Thus, the Cl^- flux is always inwardly directed, whether the animal is in freshwater of low Cl^- or on land and covered by a cutaneous surface fluid of high Cl^- concentration (993).

active uptake of Cl^- is the dominating transport mode, as found by August Krogh in 1937 (978).

Subepidermal Glands

The density of glands (see Fig. 20) is in the order of 10^3 per cm^2. Mucous glands are 5-10 times more abundant than granular glands and scattered throughout the entire surface of the body, while granular glands are relatively more abundant on the back and with a tendency of being aggregated in the neck, head, and tail regions. They are both innervated by adrenergic fibers, and secretion is stimulated by adrenergic and cholinergic agonists and humoral substances (143, 673, 957, 1226, 1309, 1699, 1733, 1892). The mucous-gland acini/skin surface-area ratio varies between 0.1 and 2.4, with the larger ratios observed in species from arid habitats (1034-1037).

Ion pathways and water channels in frog skin glands

Similar to other vertebrate exocrine glands, the ion secretion by frog skin is driven by active Cl^- transport (957). Chloride secretion by the electrically coupled acinar cells (1733) is brought about by a Na^+-$2Cl^-$-K^+ cotransporter in the basolateral membrane (143, 1226, 1231) and Cl^- channels in the luminal plasma membrane that are cAMP- (494, 1734) and Ca^{2+} activated (673, 1733) (see Fig. 25). The secondary active Cl^- transport is fueled by ATP hydrolysis at the lateral Na^+/K^+-pumps (143, 1226, 1231). A lumen-negative electrical potential and solvent drag drive the paracellular Na^+ flux. An active K^+ secretion is accomplished by the lateral Na^+/K^+ pumps in series with maxi-K^+ channels, which by single-channel recordings were shown to be co-expressed with CFTR at the luminal plasma membrane (1735) as depicted in Fig. 25. During fluid secretion, the major fraction of K^+, which is transported into the cells by the Na^+/K^+ pump and the Na^+-$2Cl^-$-K^+ cotransporter, is recirculated into the interstitial fluid by maxi-K^+ channels at the contra-luminal membrane (28). Characterized by immunofluorescence labeling, the luminal plasma membrane is also the seat of AQP-x5-like aquaporins (764). In the above studies, glandular secretions were elicited via α_1-adrenergic, β-adrenergic, muscarinic cholinergic or prostaglandin E_2 receptors. As discussed below, gland secretions serve a number of different functions, but their coupling to the above signaling pathways is unknown.

Ion composition and rate of fluid secretion

Conductance measurements of the outflow from nerve-stimulated frog skin glands (*R. temporaria*) indicated that the secreted fluid is composed of about equal amounts of Na^+ and Cl^- (989). Table 10 lists the ion composition of hormone-stimulated secretions of *R. pipiens* and *R. esculenta*. The *in vivo* Na^+ and Cl^- concentrations are a little

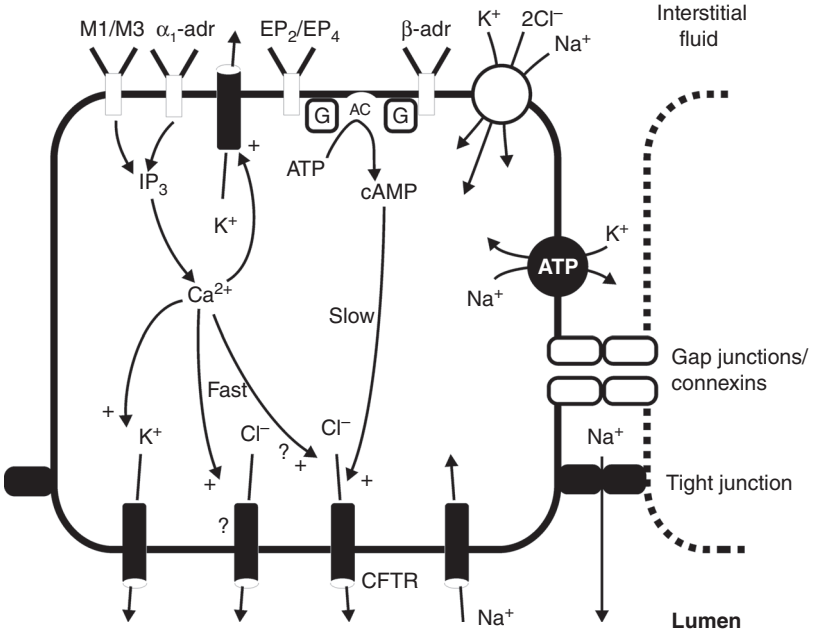

Figure 25 Ion pathways of an acinus cell of submucosal glands of frog skin. The rate of secretion is controlled by the activity of luminal Cl^- channels, which are regulated by cAMP and intracellular Ca^{2+}. The luminal K^+ channels are Ca^{2+} and depolarization activated and responsible for the relatively high K^+ of the secreted fluid. The Na^+ channels may be of importance for regulating the tonicity of the primary secretion. The model compiles results of pre-steady state flux-ratio analyses (1892), whole-cell and single-channel patch clamp studies (1733-1736) and investigations of signaling pathways with fluorescent imaging techniques (673). Modified from (1733).

Table 10 Rate of Production and Composition of Mucous Gland Secretions

Frog *in vivo* (*R. pipiens*)						
		$[Na^+]$	$[Cl^-]$	$[K^+]$	HCO_3^-	
Stimulus	Secretion rate $ml \cdot h^{-1}$	mmol/l				pH
[1]Control	0.02±0.01	–	–	–	–	–
[1]Noradrenaline, 400 μg	0.22±0.07	82.8±2.4	64.9±1.7	19.1±1.4	14.3±1.0	7.89±0.08
[1]Aminohylline, 2.5 mg	0.36±0.04	98.0±7.5	64.0±1.5	16.8±1.8	27.5±1.6	8.68±0.06
[1]Aminophylline, 5.0 mg	0.53±0.08	90.6±5.6	69.8±6.0	16.0±0.9	31.8±2.5	8.72±0.07
[1]Blood plasma	–	104.3	75.7	3.6	28.3	7.47
[2]Control	0.16±0.04	26.5±4.6	27.0±6.1	23.5±2.8	3.5±0.4	7.56±0.04
[2]Adrenaline, 1 mg	0.34±0.02	95.4±4.4	61.9±2.0	16.8±0.8	18.4±2.5	7.65±0.06
[2]Blood plasma	–	104.8	77.0	4.6	32.0	7.47

Isolated short-circuited skin, amiloride outside (*R. esculenta*)[3]								
		J_{Na}	J_{Cl}	J_K	$[Na^+]$	$[Cl^-]$	$[K^+]$	
Stimulus	Secretion rate $\mu l \cdot h^{-1} \cdot cm^{-2}$	$nmol \cdot h^{-1} \cdot cm^{-2}$			mmol/l			Sum mosmol/l
PGE_2, 2 μM	2.16±0.49	130±20	289±45	33±3.6	60.3±9.2	134±21	15.3±1.7	209
+ theophylline, 1 mM	5.51±1.04	338±32.4	606±54	69±11	61.4±5.9	110±9.8	12.5±2.0	184
Serosal fluid	–				115	118	2.5	240

[1](1934). [2](247). [3](143).

lower than the concentrations of the extracellular fluid while that of K^+ is significantly above extracellular K^+, which are characteristics also of the gland secretion by the isolated skin and of primary secretions of other vertebrate exocrine glands. A major difference is the relatively high concentration of Cl^- *in vitro* (Table 10), which is due to the isolated skin being short-circuited; Cl^- was carrying the major fraction of the short-circuit current, which matched the electrical charge flux carried by the simultaneously measured fluxes of Na^+, K^+ and Cl^-. The advantage of this method is that the ion flow (the short-circuit current with epidermal ENaC blocked by amiloride) and the fluid movement are measured simultaneously with good time resolution. Initially, stimulation elicits large transient current and fluid flows followed by smaller steady-state flows maintained for several hours (143). The *in vivo* studies of Table 10 with mean secretion rates ranging from 0.22±0.07 to 0.53±0.08 $ml \cdot h^{-1}$ were obtained in *R. pipiens* weighing 30-35 g (247). The surface area of similar-sized *R. esculenta* is about 133 cm^2 (975). Using this number, *in vitro* rates listed in Table 10 correspond to whole-body rates of 0.29±0.07 and 0.73±0.14 $ml \cdot h^{-1}$, respectively, which are comparable with rates measured *in vivo*.

Function of skin gland secretions

Besides diffusible electrolytes, the mucous gland secretion may contain glycosylated mucins and mucopolysaccharides. The bioactive material produced by the granular glands consists of neuropeptides, antimicrobial peptides, lysosomes, and antibodies, which are spread over the body surface in the cutaneous surface fluid (CSF). By covering the surface of the animal, the secretion keeps the skin moist, which is important for skin respiration and for preventing desiccation of the epidermal cells. CSF also protects against bacterial infection and entry of molds, and for making the body slippery, which helps the animal escape from predators (246, 456, 1054, 1570,

1639, 1756, 1918). Due to a particular abundance of mucous glands in the abdominal seat patch of terrestrial toads, Hillyard (764) hypothesized that mucous glands of this region are of significance for validating the quality of a hydration source prior to cutaneous drinking. It has been suggested that gland secretion in some species serves evaporative cooling (180, 235, 592, 905, 1052, 1057, 1676).

The hormone-stimulated gland secretion is near-isoosmotic or hypoosmotic with the body fluids (Table 10). Thus, if the cutaneous surface layer is maintained by gland secretion, the diffusion flux of water across the epidermis cannot be in the direction from the body fluids to the surface of the skin (993). A recent study verified this notion by measuring directly the osmolality and the Na^+ and K^+ concentrations of CSF and the lymph in *R. esculenta* subjected to raised environmental temperature or isoproterenol injection (1001, 1002). The osmolality of CSF sampled *in vivo* was 181±8 mosmol/kg at 30-34°C and 191±9 mosmol/kg in isoproterenol injected frogs at 15-23°C, while the average osmolality of the lymph was 249±10 mosmol/kg (see Fig. 26). Beyond being governed by the transepithelial osmotic gradient, the inward flow of water is coupled with the inward-active Na^+ flux (1313, 1314), probably driven by a slightly hyperosmotic paracellular compartment (1009). Therefore, in amphibians covered by a cutaneous surface fluid, the source of EWL is the fluid secreted by the mucous glands, and not water diffusing through the skin as hitherto assumed. This physiological mechanism coping with the unavoidable evaporation in amphibians prevents desiccation of the epidermal

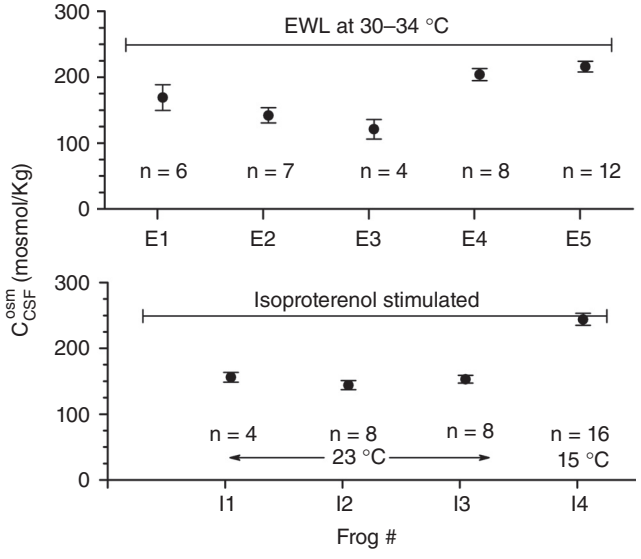

Figure 26 Osmolality of the cutaneous surface fluid (CSF) of *R. esculenta* (1001, 1002). Number of samples indicated by n is shown for each animal analyzed. *Above*: CSF samples from animals during evaporative water loss (EWL) at 30-34°C ranged from 101 to 253 mosmol/kg (mean ± s.e.m., 181±8 mosmol/kg). *Below*: The osmolality of CSF samples from isoproterenol-treated frogs at 15 and 23°C ranged from 123 to 281 mosmol/kg (mean±sem, 191±9 mosmol/kg). Horizontal bars indicate lymph concentrations of 10 frogs, 249±10 mosmol/kg (range, 228-330 mosmol/kg).

cells. Paragraphs below discuss further adaptations to terrestrial environments, such as morphological skin specializations and behavior of importance for reducing water loss by evaporation.

Turnover of the Cutaneous Surface Fluid: Functional Coupling of Gland Secretion and Epidermal Ion Uptake

If ions were not reabsorbed via the epidermal epithelium, evaporation of CSF would lead to an increase in its osmolality. In a study of *R. esculenta* of an average body mass of 43 g and a mean body surface area of 117 cm^2, Rey observed a water loss 570 $mg \cdot h^{-1}$ or 1.35 $nl \cdot cm^{-2} \cdot s^{-1}$ by evaporation (1529). With a NaCl concentration of CSF $\leq$ 100 mM, a CSF height of 8 μm and no reabsorption of ions, after no more 8 min the NaCl concentration would approach 527 mM, corresponding to a nominal osmolarity of 1054 mosmol/l. The estimate depends on the height of CSF tentatively assumed similar to the airway surface fluid of about 8 μm (1731, 1808, 1987). This might be an underestimation since the outer cornified layer of frog and toad skin is freely equilibrating with the outside medium (1543, 1545). If, on the other hand, the cornified layer confines the thickness of CSF, it would be no more than about 5 μm. Independent of the height of CSF, in the above example maintenance of a near-isotonic concentration of 100 mM NaCl would require absorption fluxes of Na^+ and Cl^- of 135-$pmol \cdot cm^{-2} \cdot s^{-1}$. Numerous studies have shown that fluxes of this magnitude of Na^+ by the principal cells (active transport) and of Cl^- by MR cells (passive transport) are in the lower range of the capacity of the epidermal transport systems at physiological skin potentials. Figure 27 summarizes the functional coupling of mucous gland secretion and epidermal reuptake of ions and water.

Regulation of osmolality and volume of cutaneous surface fluid

The function of CSF discussed above rationalizes the enigmatic finding that the Na^+ permeability of the apical plasma membrane of the principal cells of frog skin is sensitive to small osmolality differences across the epithelium (202). Thus, the Na^+ uptake was stimulated significantly by increasing the outside osmolality (π_o) by < 10%, while a bilateral increase keeping the osmolality of the serosal fluid (π_{serosa}) equal to that of the outside solution ($\pi_o = \pi_{serosa}$) had little if any effect. If the osmotic gradient was reversed ($\pi_o < \pi_{serosa}$), the apical Na^+ permeability decreased. Similar effects on the Na^+ permeability were obtained if the external osmotic displacement was obtained with NaCl, sucrose, KCl and DMSO, showing that it is the osmolality gradient that regulates the apical Na^+ permeability, independent of how the gradient is established. This type of regulation of the active Na^+ flux and the parallel change in the passive flux of Cl^- via the electrical gating mechanism discussed above would enable effective control of the ion content of the small volume of CSF. The

sensing mechanism(s) and the transduction pathways leading to the aforementioned activation of ENaC are unknown.

With toad skin epithelium (*B. bufo*) bathed bilaterally with Ringer's solutions of identical composition, β-adrenergic receptor stimulation resulted in an inward transport of near-isotonic fluid (1314). This is accomplished by cAMP activation of Cl^- channels of MR-cells (2002) and ENaCs of principal cells (87) associated with mobilization of AQP2a isoform water channels into the apical plasma membrane of the principal cells (1364). The function of this type of fluid transport would be to regulate the volume (height) of CSF at reduced EWL, for example, at low skin temperature and/or high relative humidity. Thus, during evaporation of water into the atmosphere, it is conceivable that the volume and composition of CSF are maintained by a balance between fluid secreted by subepidermal glands and water and ions absorbed by the surface epithelium, which is inferred also from the direct measurements of CSF in Fig. 26.

Energy expenditure associated with turnover of cutaneous surface fluid

With reference to Fig. 27 depicting the secretion-reabsorption couplings of ions and water in amphibian skin under terrestrial conditions, the energy expenditure associated with the turnover of CSF can be estimated by considering the oxygen consumption associated with Na^+ absorption by the epidermal epithelium and Na^+ secretion by the glandular epithelium, respectively. The active transport by the epidermal cells of 18 mol Na^+ is associated with an uptake of 1 mol O_2 (1025, 2071). This number, which is an average of measurements spanning a large range, is in accordance with 3 mol Na^+ transported for 1 mol ATP hydrolyzed by the $3Na^+/2K^+$ ATPase and a mitochondrial P/O ratio of 6. Owing to Na^+ recirculation in isotonic fluid secretion, the above stoichiometry of 18 Na^+/O_2 was estimated to be reduced to 3.4 Na^+/O_2 (1892). Returning to Rey's study (1529) mentioned above with the above Na^+/O_2 ratios, the secretion and the reabsorption of 135 $pmol \cdot cm^{-2} \cdot s^{-1}$ by the 42.6 g frog of a surface area of 117 cm^2 would require an O_2 consumption of $\sim$22 $\mu mol \cdot h^{-1}$. The O_2 uptake of similar sized frogs is about 145 $mmol \cdot h^{-1}$ (975). Thus, the energy expenditure associated with the turnover of CSF is insignificant compared to the frog's total energy metabolism.

Mechanism and physiological significance of uphill water transport by the epidermal epithelium

Skin preparations bathed bilaterally with Ringer's solution transport water in the inward direction (1517) at a rate given by the active Na^+ flux (1313, 1314) (conf. the section above on "Biophysical Concepts in Osmoregulation"). Water absorption still prevails if the external fluid is hypertonic provided the osmolality does not exceed the epithelium's capacity of uphill water transport. Thus, the water absorption indicated in Fig. 27 would proceed at moderate hypertonicity of CSF. In the semi-aquatic *R. esculenta*, water flow stopped when the outside osmolality was raised by $\Delta C_{rev} = 15.5 \pm 3.0$ mosmol/kg above that of the Ringer's osmolality (1313), while in the terrestrial *B. bufo*, $\Delta C_{rev} = 28.9 \pm 3.9$ mosmol/kg (1316). With the intercellular spaces constituting an osmotic coupling

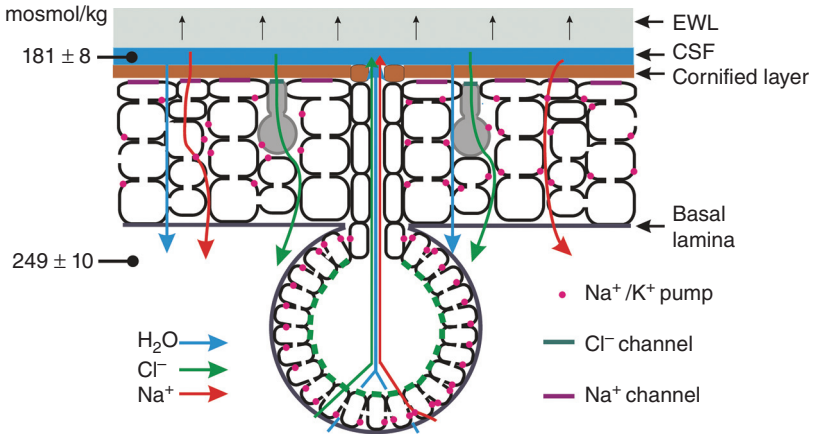

Figure 27 Proposed functional coupling of ion and water transport by subepidermal glands and epidermal epithelium of anuran skin; after E.H. Larsen (993). Two MR cells are shown in grey. On land, the skin is kept moist by the cutaneous surface fluid (CSF) produced by the mucosal glands. Water evaporates from the surface fluid (EWL), and the surplus of Na^+ is absorbed by principal cells (active transport) and Cl^- by γ-MR cells (passive transport). The volume of CSF is maintained by the balance between fluid secretion by subepidermal glands and water reabsorption by solute coupled fluid transport and/or by osmotic water uptake across the surface epithelium. Measured osmolalities are given for the interstitial fluid (lymph) and for CSF during heat-induced evaporative water loss.

compartment (1009) and water flow through the epithelial cells, the capacity of uphill water transport would be governed by the maximal osmolality of the fluid in the maze of lateral spaces and the water permeability of the plasma membranes (1006, 1961).

If the rate of evaporation is too fast for the ion uptake mechanisms to keep pace with the water loss to the atmosphere, the osmolality of CSF exceeds the capacity of uphill fluid transport, abolishing epidermal reuptake of water.

Kidney: Pronephros

During the amphibian life cycle, two kidney generations are functional, the larval pronephros and the adult mesonephros, the latter being functional already during larval stages. Early studies provided indirect evidence for the pronephros being a larval osmoregulatory organ that eliminates water and waste products and reabsorbs substances essential for metabolism (548). The morphology and ultrastructure of the larval pronephros are outlined briefly based upon a study of *B. viridis* by Møbjerg et al. (1244). Each of the paired pronephroi consists of an external glomerulus located in the coelom and a single convoluted tubule, which opens into the coelom via three nephrostomes (2-5 nephrostomes in urodeles and an even larger number in gymnophiones [548]). The capillary loops of the glomerulus are covered by an epithelium, which corresponds to the visceral layer of the Bowman's capsule of the mesonephros. The filtration barrier is much like that of the adult kidney composed by a fenestrated endothelium, a three-layered basement membrane, and processes of epithelial podocytes. From the coelom, the primary urine is swept into three branches of the nephrostome by the anteriorly located short ciliated segments. The urine continues through three branches of the proximal tubule that merge into a common proximal tubule followed by a distal tubule and a nephric duct. The urine from each of the two nephric ducts passes through the collecting ducts of the mesonephros before flowing into the cloaca. Intercalated (MR) cells are lacking in the pronephric duct, but are present in the collecting duct and in the nephric duct in larvae of a body length of 14 mm (*B. viridis*). A well-developed brush border of the proximal tubules with apical endocytotic apparatus and basolateral invaginations points to ultrastructural specializations for vectorial transport of ions and water. Functions of the fully differentiated pronephric duct of the *A. mexicanum* were addressed recently (715). Impalements with microelectrodes of single microperfused tubules of larvae stage 46-54 indicated negative intracellular potentials averaging between −75 mV and −80 mV. Peritubular and luminal ion substitutions revealed a relatively large basolateral K^+ conductance and Na^+ and K^+ conductances in the luminal membrane. Immunolabeling and confocal laser scanning microscopy localized Na^+/K^+ ATPases to the highly invaginated basolateral membranes. Thus, it seems that the pronephric duct is important for diluting the urine formed by ultrafiltration and for excreting K^+, and that both of these functions are energized by ATP hydrolysis at Na^+/K^+ pumps. Further evidence for rheogenic Na^+ reabsorption was obtained in a study of the pronephric duct of the Japanese black salamander, *Hynobius nigrescens*, in which ENaCα was immunolocalized at the luminal membrane of epithelial cells that expressed the Na^+/K^+ ATPases at the peritubular membrane (1882). A putative NHE3 isoform of the Na^+/H^+ exchanger has been immunolocalized at the luminal membrane of the anterior portion of the pronephric duct in the pronephros of this species, indicating a luminal uptake mechanism for Na^+ coupled to proton secretion (981).

Kidney: Mesonephros

The number of nephrons of each kidney depends on species with no more than 60 in the small African dwarf frog, *Hymenochirus boettgeri* of a body mass of 1.3 g and 8,000 in large Colorado river toad, *B. alvarius* of a body mass >200 g (1884). In *B. bufo*, the number in each kidney is ~3,000 (1245), while it is ~1,700 in the fossorial African caecilian *Geotrypetes seraphini* (1243). As indicated in Fig. 28, the nephron of the metamorphosed amphibian consists of a Malpighian corpuscle (the Bowman's capsule surrounding a glomerulus), a ciliated neck segment, a proximal tubule, a ciliated intermediate segment, an early and a late distal tubule, and a collecting tubule, which opens into a collecting duct

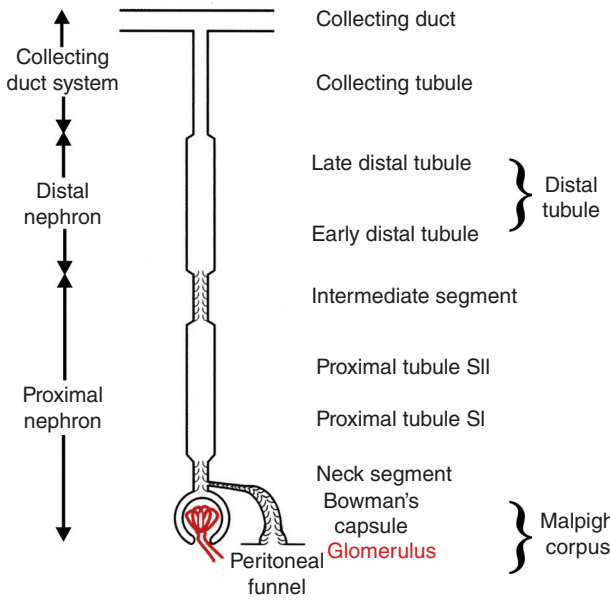

Figure 28 Generalized diagram of amphibian nephron with nomenclature indicated. The proximal nephron comprises Malpighian corpuscle, the ciliated neck segment, the proximal tubule and the ciliated intermediate segment. The subdivision of SI and SII segments is based on dimension of brush border and basolateral infoldings. The Distal nephron comprises the early and late distal tubule, the collecting tubule and the first unbranched portion of the collecting duct system. As shown here, in urodeles and caecilians, the peritoneal funnel connects the proximal nephron with the coelom. In anurans, this connection is lost and the peritoneal funnel opens into the peritubular vessel surrounding the nephron. From (1243).

shared with other nephrons. In salamanders and caecilians, a ciliated peritoneal funnel opening close to the glomerulus connects the lumen of the nephron with the coelom. In anurans, this connection between the nephron and the coelom is lost, and the peritoneal funnel opens into the peritubular vessel surrounding the nephron. Renal arteries from aorta supply blood to the kidneys, which also receive vessels from the renal venous portal system (257, 314, 1070, 1243, 1245, 1596, 1598, 1883). The anatomical description of distal nephron segments of different amphibians has resulted in several synonyms, which are discussed in (1245). Evolution of the amphibian nephron is considered in (1243).

The literature on renal functions of amphibians is reviewed in (162, 376, 764, 1670). The discussion below focuses on two major functions related to the environmental physiology of amphibians. In freshwater, the kidneys produce a large volume of highly diluted urine for eliminating the water taken up by osmosis across the skin. In terrestrial habitats, the kidneys produce a small urine volume. Thus, even if amphibians cannot concentrate urine, renal water conservation is well developed and after a loss of only a few percent of body water, they can become anuric. The urine volume depends on the rate of primary urine production and the tubular processing of water.

Glomerular filtration

The glomerular filtration rate (GFR) is the major determinant of the rate of urine production (544, 586, 1624), which depends on the number of active nephrons, the net glomerular filtration pressure, and physical properties of the filtration barrier, that is, total area, filtration coefficient and fractional filtration rate (1432, 1434). Three studies of single-nephrons of freshwater-acclimated urodeles and anurans indicated single-nephron filtration rates (SNGFR) between 0.1 $\mu l \cdot h^{-1}$ and 1 $\mu l \cdot h^{-1}$ (609, 1432, 1922). In a study of Amphiuma kidney, Persson and Marsh (1433) demonstrated a link between flow dependent entry of Cl^- into the epithelial cells of the early distal tubule and SNGFR. This observation and results of previous studies (1434, 1727) suggest that tubulo-glomerular feedback constitutes an intrarenal mechanism by which blood flow in afferent arterioles and GFR is regulated by the flow rate in the distal tubules. The reduced GFR seen upon transfer from freshwater to either terrestrial conditions (1624, 1669, 1765, 1876) or increased salinity (1192, 1632, 1678) is caused by a reduction of the number of filtering glomeruli, presumably with little or no contribution of glomeruli that filter continuously at reduced rate (376). The downregulation of GFR and the increased tubular water reabsorption upon water restriction operate fast and are independent of prior desiccation or extracellular volume loss (1435, 1876). The posterior pituitary ADH decreases GFR and increases tubular water reabsorption (21, 95, 100, 587, 1614). There are additional as yet unidentified regulatory mechanisms involved, which is indicated by the observation that surgical elimination of the neurosecretory system that produces the antidiuretic hormone was without effect on anuran water metabolism (873, 874).

Proximal tubule

The primary urine is an ultrafiltrate of plasma formed by the fenestrated endothelial cells of the glomerular capillary and the foot processes of the epithelial podocytes, which constitute the visceral layer of Bowman's capsule (1938, 1979). The proximal tubule belongs to the class of "leaky epithelia" with an intercellular (junctional) resistance, which is between one and two orders of magnitude less than the transcellular resistance (159, 610). In anurans and salamanders, the transepithelial potential difference (V_T) is lumen negative, varying numerically from 3 mV to 15 mV governed by the active Na^+ flux and a low resistance paracellular 'shunt' (106, 160, 608, 1777, 1985). The epithelial cells are furnished with brush border, basolateral infoldings and an apical endocytotic apparatus (314, 1185, 1243, 1245, 1597). The Na^+/K^+ ATPase is abundantly expressed in plasma membranes lining the lateral intercellular spaces and basolateral infoldings (1183-1185). The major fraction of the ultrafiltrate including glucose (1921), amino acids (1367) and proteins (1492) is reabsorbed in the proximal tubule as isotonic fluid coupled to the transport of Na^+ (2013), which is active (1748). Water can flow across the epithelium through both the cellular ("translateral") and the paracellular ("transjunctional") pathway (1866), as indicated in Fig. 1B. The major influxes of Na^+ and Cl^- across the brush border membrane are mediated by Na^+/H^+- and Cl^-/HCO_3^- exchange mechanisms, respectively (155, 1646). In addition to the Na^+/K^+ pump flux, the exit of Na^+ across the peritubular membrane takes place as cotransport with HCO_3^- of a stoichiometry estimated to be 3 HCO_3^- : 1 Na^+ (154, 1095). The exit of one Cl^- across the peritubular membrane is in exchange with one Na^+ and two HCO_3^- from the peritubular side (677, 1092). In parallel with this mechanism, there is evidence also for Cl^-/HCO_3^- exchange that would transport Cl^- into the cell from the interstitial fluid (473, 2060). Further to serving transtubular H^+ and HCO_3^- fluxes, the above acid-base transporters are supposed to regulate intracellular pH (154, 155). In *Necturus*, a cell negative basolateral membrane potential of about −70 mV is governed by a relatively large K^+ conductance of the peritubular plasma membrane (608).

Early distal tubule: Diluting segment

Based on electrophysiological criteria, Stoner (1777) identified two different segments of the distal tubule in frogs, toads and salamanders; an early distal tubule, denoted the diluting segment of a lumen-positive V_T, and a late distal tubule, sometime referred to as the junctional segment, of a lumen-negative V_T. In *Ambystoma*, the functional heterogeneity could be correlated with morphology and ultrastructure at cell level (768). As indicated by the name, in the diluting segment ions and water are separated by reuptake of Na^+ and Cl^- through the

tubular epithelium of a relatively low water permeability (678, 679, 1777). Thus, it is in this segment and in the collecting duct system that the osmolality of the tubule fluid is reduced in freshwater-acclimated animals. The magnitude of the lumen-positive V_T seems species dependent, varying from +5 and +8 mV in *B. marinus* and *Amphiuma means*, respectively, to +22 mV in *Triturus alpestris* (1433, 1777, 1818). Flux studies and intracellular ion activity measurements in anurans and salamanders indicated that the uptake mechanism in the luminal plasma membrane is an electroneutral cotransporter of a stoichiometry of 1 Na^+:1 K^+: 2 Cl^- (1351, 1352, 1354-1356, 1777). The lumen-positive V_T is due to a luminal membrane potential (cell negative) that exceeds numerically the potential difference across the peritubular plasma membrane (1353). This owes to a relatively large passive K^+ conductance of the luminal plasma membrane, which returns a major fraction of K^+ taken up by the cotransporter. In *Ambystoma*, the luminal K^+ conductance and a Na^+/H^+ exchange mechanism were stimulated by K^+ loading (1350, 1357). In a study combining intracellular potential and cell volume recordings, Guggino (676) identified two cell types of the early distal tubule of *Amphiuma*: one cell type in which both Cl^- and K^+ move across the peritubular membrane by electrodiffusion, and another cell type in which the two ions exit across the peritubular membrane in an electroneutral fashion via a K^+-Cl^- cotransporter. In a subsequent review, Guggino et al. (678) concluded that functionally the amphibian early distal tubule has much in common with the mammalian thick ascending limb.

Late distal tubule

The functional organization of the late distal tubule has been more difficult to study because the electrophysiological properties change along the tubule. Following Stoner's definition, the late distal tubule is a "tight" epithelium with an upper-limit transepithelial resistance of about 1,200 $\Omega \cdot cm^2$ and a lumen-negative V_T averaging from −6 to (occasionally) about −40 mV with more negative values recorded toward the collecting tubule (26, 1469, 1777, 1818). Anagnostopoulos and Planelles (26) measured the electrochemical potential differences of Cl^-, K^+, Na^+ and H^+ across the peritubular and luminal plasma membranes of *Necturus in vivo*, which can be summarized as follows. With the renal surface superperfused by Ringer's solution of pH = 7.4, the ion activities of the peritubular capillary blood were (mM), 71.0 Na^+, 2.5 K^+ and 70.5 Cl^-, pH = 7.37. With the urine stemming from the diluting segment, the tubule cells of the late distal tubule maintained activities of the luminal fluid of about (mM) 9 Na^+, 2.5 K^+ and 12 Cl^- and a pH of 6.5 at an average V_T of −14 mV. For Na^+, K^+ and Cl^-, the measured transepithelial electrochemical gradients were opposite to the established inwardly directed net transports, which therefore would have to be transcellular and active. The steady-state distribution of protons indicated active secretion of this ion. With an apical membrane potential of about −60 mV (cell negative) and the following intracellular activities (mM), 9.0 Na^+, 65.8 K^+ and 5.5 Cl^-, the active step for the Cl^- flux would be at the luminal plasma membrane. The flux of Na^+ from lumen to cell is downhill and active across the peritubular plasma membrane. When K^+ is absorbed, an active K^+ uptake step is necessarily at the apical cell membrane. Protons would be actively secreted from cells into the lumen. Finally, the steady-state cellular activities of Na^+ and K^+ were compatible with a Na^+/K^+ pump in the peritubular plasma membrane. In a study of isolated microperfused late distal tubule of *Amphiuma*, Stanton et al. (1753) confirmed active acid secretion toward the luminal fluid, which occurred by an electroneutral amiloride-sensitive Na^+/H^+ exchange mechanism in the luminal plasma membrane of a socalled type I cell. Another cell type denoted the type II cell, absorbed NaCl in an electroneutral fashion, probably mediated by the parallel operation Na^+/H^+ and Cl^-/HCO_3^- exchangers, that was inhibited by the thiazide diuretics targeting the anion exchanger (1754, 1755). The type I and type II cells, respectively, were identified by a difference of the peritubular membrane-potential response to inhibitors added to the luminal perfusion solution. Neither amiloride nor thiazide affected the electrophysiological properties of the acid-secreting type I cell. In contrast, these diuretics as well as DIDS hyperpolarized the peritubular plasma membrane of type II cells. Both cell types were characterized by a fractional resistance of the luminal plasma membrane near unity, compatible with the ion fluxes through the luminal plasma membrane being electrically silent (1753). V_T of the above microperfused late distal tubule of *Amphiuma* was not significantly different from zero. As previously mentioned, V_T increases numerically toward the collecting tubule (1818), indicating that the studies of *Amphiuma* and the above studies of *Necturus*, respectively, were performed on different portions of late distal tubule. According to the literature, there is a remarkable difference between *Amphiuma* and *Necturus*. Cable analysis of the *Amphiuma* tubule *in vitro* indicated a transcellular resistance (R_T) of no more than 72 $\Omega \cdot cm^2$ (1753), which is more than an order of magnitude lower than that of the *Necturus* tubule studied *in vivo* (26, 1469). Because the fractional resistance of the luminal membrane of both type I and type II cells is close to unity (1753, 1755), the low R_T of *Amphiuma* late distal tubule may have been governed by paracellular ion fluxes. This, however, deserves further investigations.

Collecting duct system

The collecting tubule is the terminal part of the nephron that opens into the collecting ducts (Fig. 28). Structurally, the transition between the late distal tubule and the collecting tubule is gradual, and they have been considered together as analog segments in non-mammalian vertebrates (376). In *B. bufo*, the epithelium of the collecting tubule and the collecting ducts is heterocellular with principal cells and intercalated MR cells. The MR cells constitute about 30% of the total cell number of the collecting tubule (1245).

There are indications of functions similar to those of MR cells of the skin. Thus, double immunofluorescence staining with rabbit anti-*Bufo* ENaCα antibody and anti-vacuolar-type H^+-ATPase antiserum showed luminal expression of the Na^+ channel at the principal cells and the proton pump at the intercalated mitochondria cells, respectively, of the Japanese black salamander (1882). Like the principal cells of frog skin, the uptake of Na^+ across the luminal membrane of the principal cells is passive via channels with ENaC-phenotype, that is, a small-conductance (< 10 pS) amiloride-blockable Na^+ channel. The transtubular Na^+ uptake is fueled by basolateral ouabain sensitive Na^+/K^+ pumps located in a K^+ selective peritubular membrane. With a lumen-negative V_T, the Cl^- flux from lumen to the peritubular space is believed to be passive and to pass the tubular epithelium between the cells (816, 1777, 1778, 1802, 1882). By reabsorbing Na^+ and Cl^-, the collecting duct system contributes to the dilution of the urine. Under certain physiological conditions water is supposed to be reabsorbed from the diluted tubular fluid, because AVT stimulation results in the translocation of aquaporin AQP-h2K to the luminal plasma membrane of principal cells (1365) and the reabsorption of water (575, 1397, 1613).

The collecting duct system has capacity to secrete K^+ and H^+ via cellular pathways and to participate in the regulation of the body balance of inorganic phosphate (1246, 1247, 1780, 2063). Potassium-loaded *Amphiuma tigrinum* kept in 50 mM environmental KCl upregulated (activated and/or increased the number of) maxi K^+ channels and intermediate non-selective cation channels at the luminal membrane supposed to mediate the urinary K^+ secretion in hyperkalemic animals (1779, 1781). As previously discussed, with a plasma pH of about 7.8 and a relatively higher partial CO_2 pressure (pCO_2), the plasma bicarbonate concentration in air-breathing amphibians is larger than in water-breathing animals. Yucha and Stoner demonstrated a dependence of the lumen-to-peritubular flux of HCO_3^- in the initial collecting tubule on plasma pCO_2, suggesting that tubular acid/base transporters participate in the regulation of plasma [HCO_3^-]. Because the uptake of HCO_3^- was reduced by amiloride or in Na^+-free perfusion solution, they hypothesized that a luminal Na^+/H^+ exchange mechanism is involved in the transtubular bicarbonate uptake (2063).

Urinary Bladder

The Wolffian ducts from the kidneys lead the urine directly into the cloaca, which then runs back into the unpaired thin-walled urinary bladder. The epithelium of the bladder serves the final adjustment of the ion concentrations of the urine (4, 1024, 1221, 1262) and participates in the regulation of whole-body acid-base balance (550) by a carbonic anhydrase containing minority cell type (1577), presumably MR cells. In amphibians on land, the bladder constitutes a water storage organ and during evaporative water loss, water is reabsorbed from the bladder urine (96, 101, 868, 1757). The significance of this function is illustrated by the maximal volume of water in the bladder. For example, expressed in percent of body mass the bladder capacity of aquatic amphibians like *Xenopus, Triturus cristatus* and *Necturus maculosus* is less than 5% (91, 100), as compared to 50%, or more, in fossorial species like *Cyclorana platycephalus*, *Notaden nicholsi* and *Scaphiopus couchi* (1144, 1671). Reabsorption of bladder urine is mediated by a distinct urinary bladder aquaporin, AQP-h2 at the luminal plasma membrane of principal cells (1363).

The luminal surface of the bladder wall is a semi-dual-layered heterocellular epithelium including basal cells, principal cells (also denoted granular cells) and intercalated MR cells. Goblet cells constitute a fourth cell type that contains mucous filled vesicles (425, 1409, 1643, 1786). The bladder epithelium of *Amphiuma* contains only three cell types, with the goblet cells missing. In this species, the MR cells comprise less than 5% of the total number of cells at the surface of the epithelium (1262) as compared to 15% in *B. marinus* (1643) and 25% in *R. catesbeiana* (1786). Electron microprobe analysis of the cellular content of Na^+ and K^+, respectively, and their response to perturbation of ion composition of the bathing solutions and serosal ouabain exposure indicated that all four cell types in *B. marinus* are configured for vectorial transport with apical Na^+ entrance pathways and basolateral Na^+/K^+ pumps (1544). The bladder epithelium is of the high-resistance type with a lumen-negative V_T, rheogenic active Na^+ absorption and active Cl^- absorption under short-circuit conditions (439, 1024, 1262, 1284). The active Na^+ uptake is regulated by aldosterone (348-350). By the use of a radiolabeled amiloride analogue [^{3}H]benzamil and polyclonal anti-Na^+ channel antibodies, Kleyman et al. (946) localized a Na^+ channel with ENaC phenotype at the apical plasma membrane (*B. marinus*). This was confirmed by Konno et al. by immunohistochemical localization of the α-subunit of ENaC. Unexpectedly (conf. 1544), the Na^+ channel was expressed at the apical membrane of principal cells with no staining of MR cells (962).

Aquaporins in Amphibian Osmoregulatory Epithelia

By governing the hydrosmotic permeability of the above osmoregulatory epithelia, aquaporins (AQPs) play a fundamental role in the water economy of terrestrial anurans whether the source of water is environmental water or bladder urine (1363, 1790, 1792). As indicated above, the cellular sites of amphibian analogs have been studied in some details. Generally, the transepithelial water permeability is regulated by insertion of anuran analogues of APQ2 into the apical plasma membrane of the principal cells. Water leaves the epithelial cell via the basolateral plasma membrane through constitutively expressed AQP-h3BL (14), which is an aquaglyceroporin that is permeated also by glycerol and urea (7). The analogs discussed below are specific for anurans and structurally distinct from mammalian APQ2.

Aquaporins of ventral skin epithelium

Following AVT stimulation, AQP-2h (*B. marinus* and *H. arborea*) and/or AQP-3h (*B. marinus* and *H. arborea, R. catesbeiana*, *R. japonica*, *R. nigromaculata*) are inserted into the apical plasma membrane of the outermost living cell layer of water absorbing ventral skin regions (712, 1363, 1366, 1791). Interestingly, AQP2a paralogues resided below the apical plasma membrane in hydrated, non-stimulated anurans, which might allow for fast transfer to the plasma membrane when an external water source becomes available (1366). AQP-h3-like AQPs have been denoted the "ventral skin-type" AQP because of an exclusive expression in the skin (1363).

Aquaporins of distal renal epithelia and urinary bladder

Two AQP's seem specifically associated with distal epithelia of the anuran kidney: AQP-h2K in *H. japonica* (1365), and HC-2 AQP in *H. chrysoscelis* (2076), respectively. Following AVT stimulation AQP-h2K was translocated to the apical plasma membrane of the principal cells of the collecting duct, indicating that it governs renal water reabsorption. Immunofluorescence staining for AQP-h3BL indicated that the protein is constitutively expressed at the basolateral membrane of the collecting duct principal cells (1365). Besides the pelvic skin, Tanaka's group detected AQP-h2 also in the urinary bladder, which they referred to as the "urinary bladder-type" amphibian AQP (1363) to distinguish it from the other isoform that was not found in the urinary bladder of *H. japonica* (1806).

Aquaporins of the aquatic Xenopus

The *Xenopus* AQP-x3 is a homologue of the pelvic skin-type AQPa2. However, its gene expression is attenuated at an identified post-transcriptional step (1363). Therefore, the gene is not expressed at the protein level, which explains the low water permeability of the skin of *X. laevis* (293, 2059). Another distinctive feature of the water economy of *X. laevis* is the vanishing urinary bladder volume capacity (91).

Amphibian Osmoregulation: Adaptations to Terrestrial Environments

Studies of amphibian water economy have been carried out for more than 200 years (860). The first study by Robert Townson indicated that frogs without access to a pool of free water maintain water balance under terrestrial conditions (865, 1849). The more recent study by Adolph (6) providing new quantitative information on amphibian water metabolism concluded: "It is evident that with respect to water balance a frog is unsuited to non-aquatic existence." Studies following Adolph's and discussed below unanimously showed that, contrary to his conclusion, amphibians are highly and successfully adapted to a terrestrial life.

Evaporative water loss

In a study of European anurans (*R. esculenta*, *R. temporaria*, *Bombinator igneus*) and urodeles (*Triturus taeniatus, T. cristatus*), leading to his *Thirty Nine Theses on the Water Economy of Amphibians and the Osmotic Properties of the Amphibian Skin*, Ernest Overton concluded that water evaporates as from a dilute salt solution depending on temperature, relative humidity and surface area (1387). This was also the finding of Edward F. Adolph and Pierre Rey, respectively, who concluded that the skin does not offer any resistance to water evaporation into the atmosphere (6, 1529). This notion was further supported by the observation that live and skinned frogs lost water by about the same rate (6) and similar to the rate of water loss by evaporation from replicas made of agar with the same shape and surface area as live animals. Although this view was modified subsequently by studying species of a variety of habitats (1053, 1744, 2045), it has been accepted generally that the evaporative water loss in amphibians is caused by an unavoidable diffusion of water through the (more or less) water-permeable skin. However, the methods and experimental protocols of the above classical studies on live animals (6, 1387, 1529) do not allow for the distinction between CSF produced by the glands versus a transcutaneous diffusion of water as the source of EWL. As discussed above on cutaneous transport mechanisms, the slightly hypotonic CSF (1002) implies that the transcutaneous flow of water is directed from the cornified side to the interstitial space driven by the shallow osmotic gradient and by solute coupled fluid transport (Fig. 1B). Therefore, EWL stems from fluid produced by subepidermal glands. In a comparative study of 13 anuran species from diverse habitats, Lillywhite and Licht (1054) observed that curarized anurans exposed to increasing temperatures from 18°C to 34°C discharged a clear non-viscous fluid that formed a liquid surface film. With some variation, discharges were more frequent at higher body temperatures. These observations applied to frogs (*Hyla cadaverina, Litoria aurea, Rana boylei, Afrixalus brachycnemis*), but not to *Bufo woodhousei*; all five species mentioned exhibit in nature basking behavior in sunlight. Nor did curarized nocturnal (*H. punctatus, Phyllomedusa tarsius*), fossorial (*Kaolula pulchra, Scaphiopus bombifrons, Ensatina eschscholtzi*) and aquatic species (*Pipa pipa, Ooedizyga lima*) respond by the above type of gland secretion to increased temperature in the laboratory (1054). The individual discharge event is the result of emptying the acinus of a single submucosal gland by contraction of the thin layer of myoepithelial cells (143). The sustained flow of ions and water through the acinus epithelium cannot be recorded by the above method.

As discussed above, the cutaneous surface layer seems to constitute a regulated external physiological compartment from which evaporation of water takes place. Thus, the terrestrial environment imposes a demand for a relatively fast water turnover with potential danger of excessive and irreversible water loss. Owing to the larger surface-area to body-size ratio, generally obeying the isometric exponent of about

0.67 (860), this danger is even more severe for small animals. Numerous studies of amphibian water economy in various terrestrial habitats have disclosed a diversity of mechanisms evolved that would prevent this fatal situation to occur. The monograph by Hillman et al. (759) contains an extensive review of the large literature on the issue. A brief summary of major principles illustrated by examples is given below.

Behavioral reduction of body water loss

In the field, EWL rates are strongly influenced by relative humidity, air temperature, wind speed and by absorbed radiation, but normally not by substrate temperature (1850). As an exception, the euryhaline leptodactylid frog *Thoropa miliaris* living in rocky shores maintains its body temperature 3-4°C above air temperature by the rocky surface warmed by the sun (2). Among the above environmental parameters, temperature and water are probably those that most directly affect the behavior of terrestrial amphibians. Several species are nocturnal and by diurnal activity patterns, EWL is reduced by the animal seeking microhabitats of low temperature, high relative humidity and low wind speed (412, 780, 1010, 1197, 1478, 1670, 1767). Anurans living in arid habitats escape the dehydrating environment by burrowing and returning to the surface during rainfall (255, 256, 435, 780, 896, 1193, 1195, 1587, 1652-1654). Water-conserving posture constitutes another behavior serving a reduced EWL. To reduce the exposed surface area, the body and chin may be pressed to the substrate and the limbs tucked under the body (759, 1478, 1829). Other examples include tight coiling of the body and tail in elongated salamanders (1513) and aggregation of individuals into groups for reducing the "surface-area to body-mass" ratio with a tendency to sit on top of each other with increasing numbers in the group. The crowding behavior results in a lower EWL than the individual animal otherwise would experience (19, 869, 1528, 1932).

Waterproofing barriers

The skin resistance to evaporative water loss, r_{EWL}, was introduced for quantifying and comparing EWL in the laboratory of animals from different terrestrial habitats (83). It has the dimension of $s \cdot cm^{-1}$ and is calculated as the reciprocal of EWL per cm^2 of cutaneous surface area per unit of vapor pressure difference between the animal and the surrounding air at a specified skin temperature,

$$r_{EWL} = \frac{\Delta\rho}{EWL}$$

If EWL is in $g \cdot cm^{-2} \cdot s^{-1}$, $\Delta\rho$ is the vapor-density difference between the animal and the surrounding air in $g \cdot cm^{-3}$. Because of a small solute concentration, the body fluid is assumed saturated with water vapor at the given temperature. According to Spotila and Berman (1744), r_{EWL} is partitioned into the internal resistance, r_i, and the external boundary-layer resistance, r_b, *i.e.*, $r_{EWL} = r_i + r_b$. EWL increases with the activity of the animal (724) and r_{EWL} is therefore measured in immobilized animals (mechanically, curarized or pithed) with emptied urinary bladder, blotted dry with a paper towel, and placed in a test section of a wind tunnel of relatively fast laminar air flow (100-200 $cm \cdot s^{-1}$). For estimating the boundary resistance, replicas of the animals are made in 3% agar with $r_i = 0$, so that $r_{EWL} = r_b$ (1744, 2045). In a recent study, Lillywhite collected and discussed a large number of skin resistances to EWL of amphibians, birds and mammals (1053). The resistance to evaporation of a free water surface is zero. In moist skinned anurans adapted to mesic habitats (*Bufo, Rana, Scaphiopus*), r_i is close to zero and lower than r_i of arboreal frogs, with values ranging from 1.4 to 2.7 $s \cdot cm^{-1}$ (2044, 2045). A comparative study of 25 species of Northern Australian frogs belonging to four different families reported skin resistances to EWL covering the range between 0.6 $s \cdot cm^{-1}$ and 63.1 $s \cdot cm^{-1}$. The frogs were from aquatic, terrestrial and arboreal habitats, and there was a clear correlation between ecological habitat and r_{EWL} (1851, 2061). The authors suggested that terrestrial and arboreal species displaying higher resistances to water loss take advantage of high environmental temperatures where growth and locomotory speed is higher, without the risk of desiccation. Some frogs are classified as "waterproof" because their skin resistance to EWL of 300-500 $s \cdot cm^{-1}$ compares to reptiles with thick non-hydrated stratum corneum (235, 450, 592, 1053, 1100, 1668, 2015, 2016, 2045). The mechanism seems to be different for the different species (759). In the South American tree frog *Phyllomedusa*, the glands secrete a waxy material of predominantly wax esters, free fatty acids and hydrocarbons (145, 623), which is spread over the body surface (1199). Several studies aimed at the mechanism of intermediate skin resistances to EWL, but with no clear conclusion (1053).

During periods of aestivation in drying soils, some anurans and urodeles become encapsulated in a cocoon formed by moultings without casting off the sloughs. Lipids and proteinaceous materials are deposited between the multiple superimposed cornified layers (255, 297, 1198, 1586, 2014). The resistance to water diffusion increases with the number of layers added. In the South American frog from subtropical and tropical dry forests, *Lepidobatrachus llanensis*, r_i increased from ~0 $s \cdot cm^{-1}$ in the uncocooned frog to 116 $s\ cm^{-1}$ in an 38-layered cocoon, which was reached after 35 days of aestivation (1196). Thus, the shedding intervals were about 1.6 day as compared to about 5 days in other anurans at temperatures at about 20°C (1011). In *Cyclorana australis*, the cocoon impeded water loss to the soil of a significantly lower water potential than the animal (1531). During a five-month aestivation period, the lymph concentration of Na^+ was kept almost constant at about 100 mM, while the urea concentration increased from 12 mM to 85 mM. The urea concentration of bladder urine increased as well, but not as much as that of the body fluids, thus maintaining the osmotic gradient for absorption of water from the bladder (1532).

Some amphibians have developed distinctive surface sculpturing like cutaneous grooves, channels, warts and prominent elevations denoted verrucae with so-called tubercles and conical short spines (402, 488, 1840). These macroscopic liquid-air interfaces are sites of vaporization and condensation. It is not known how they affect the boundary layer resistance of evaporation. The question may be a point of departure in future studies.

Water uptake by cutaneous drinking

Amphibians do not drink by the mouth, but take up water through the skin. This occurs from a free water surface (102, 105, 865, 1849) or from soil water if the water potential is higher than that of the body fluids (723, 762, 780-782, 1194, 1530, 1743, 1923). In species of Bufonidae, epidermal sculpturing increases the rate of rehydration because capillary forces drive water from the hydration source over the body surface, which increases the area engaged in water uptake (293, 1055). The ventral pelvic skin of semiterrestrial anurans denoted the "seat patch" is hypervascularized and is most important for cutaneous drinking (66, 294, 295, 369, 1195). Comparative studies showed that the pelvic region of *B. bufo*, *B. alvarius, R. esculenta, R. arvalis,* and *R. temporaria* is more vascularised than the pelvic region of the aquatic *X. laevis* (293, 1579). The rate of rehydration increases with the density of vascularization (293) and arterial perfusion with Ringer's solution of the pelvic skin stimulated by AVT *in vitro* enhanced the osmotic water influx from artificial tapwater by maintaining a large osmotic pressure gradient across the epidermis (294). Blood flow through the pelvic region increased *in vivo* when dehydrated *B. bufo* and *B. woodhouseii* were placed on a wet substrate (1911, 1912), but not on a dry substrate (1911). Thus, cutaneous drinking is associated with enhanced osmotic permeability of the epidermal cells and an increased blood flow through the capillaries of the seat patch region. Illustrated above by the hydration status of the animal and an appropriate hydration source, respectively, cutaneous drinking is controlled by intrinsic as well as extrinsic factors (767).

Intrinsic factors Dehydration constitutes an intrinsic stimulus for cutaneous drinking, and AVT is widely accepted as a messenger. This is because injection of neurohypophyseal extracts or AVT results in positive body water balance and enhanced hydrosmotic permeability of the skin and because the plasma concentration of AVT is increased in dehydrated animals (21, 22, 92, 93, 100, 209, 212, 515, 727, 728, 861, 961, 1327, 1328, 1839). The neurohypophyseal peptides, hydrins 1 and −2 also stimulate the water permeability of the skin, to a level similar to that of AVT stimulation (5, 1364). However, dehydration of *B. bufo* and *R. temporaria* increased the rate of rehydration much more than did supramaximal doses of neurohypophyseal extracts or AVT (862, 872). Furthermore, surgical elimination of the neurosecretory system was without effect on the water metabolism of *B. bufo*, which still displayed enhanced water uptake in response to dehydration (873, 874). This would imply that neither AVT nor the hydrins necessarily play decisive physiological roles in regulating water balance and osmotic permeability of the skin. The water permeability of the skin monitored directly by the water flow *in vitro* or by the rate of rehydration in dehydrated animals is also under control by the adrenergic nervous system (761, 1499, 1514, 1738, 2057). Hoff and Hillyard (779) have shown that angiotensin II increases the rate of water uptake in fully hydrated toads (*B. punctatus*) that could be prevented by injection of sarasalsin, which is an inhibitor of the angiotensin type-1 receptor. Thus, several hormones have capacity to stimulate the cutaneous water permeability. Their physiological role and interactions with other intrinsic factors in the water economy of amphibians remain to be fully established. Interestingly, the urinary bladder filling by itself constitutes a stimulus for cutaneous drinking. Thus, emptying the bladder of toads in water balance (*B. woodhouseii*, *B. bufo*) initiated drinking behavior and cutaneous water uptake (868, 1855).

Extrinsic factors and drinking behavior Toads detect the quality of a hydration source with receptors on their feet (1767, 1768). Initially, the toad keeps the ventral skin raised above the substrate so only the feet are in contact with the substrate. According to Hillyard, the "seat patch up" (SPU) posture may last for periods of seconds or minutes before the toad allows the ventral skin to contact the substrate. In the "seat patch down" (SPD) posture, the ionic or osmotic properties seem to be evaluated. If the substrate is accepted, the water absorption response (WR) is initiated where the hind limbs are abducted and the ventral skin is pressed to the hydration surface. Dehydrated *B. punctatus* avoided a hydration source made hyperosmotic by urea (195) or by NaCl (778), suggesting that receptors in the skin detect the osmolality of the substrate. Hillyard and coworkers analyzed the sensory response in studies that integrated behavioral observations with membrane potential recordings from principal cells of the skin, and measurements of the electrical activity of efferent branches of neural nerves innervating the ventral skin. The duration of WR was largely unaffected by increasing the NaCl concentration of the hydration source as long as it was hypoosmotic or not too hyperosmotic to the body fluids. Superfusing the isolated skin epithelium with a 250 mM NaCl-solution (∼500 mOsm), which suppresses WR *in vivo*, depolarized the basolateral membrane and induced trains of neuronal activity of spinal nerve V and VI. The electrical responses vanished if 10-μM amiloride was added to the perfusion solution, providing evidence for a Na^+ influx through ENaC as the early event in the sensing of the quality of the hydration source (763, 766, 1269). In a concomitant paper (1789), they studied the ability of different salts to suppress WR behavior at equivalent concentrations (250 mM) of different Na^+-salts. Using the hydration time (i.e., the sum of SPD and WR) as a measure of the tolerance, a linear increase in tolerance was observed with increasing molecular weight

of the anions tested (chloride, acetate, phosphate and gluconate). It was hypothesized, therefore, that the depolarization of the basolateral membrane is caused by an influx of Na^+ via ENaC with the loop current carried by an inward flow of anions through opened tight junctions. This was compatible with an observed large and reversible increase in the transepithelial conductance. Their hypothesis conforms to the early observation that intercellular junctions of tight epithelia reversibly become leaky if the outside is exposed to a hyperosmotic solution (298, 497, 1893), and proposes for the first time a physiological role of the "leaky state" of the skin epithelium.

Dynamic Aspects of Amphibian Osmoregulation: Coupling of Skin, Kidney, and Bladder Functions

Amphibian osmoregulation involves mechanisms primarily aimed at the volume of the extracellular fluid, and others primarily aimed at the osmolality. They are integrated, although temporarily one is given priority above the other (29, 489, 863, 864, 1225, 1769, 2065). Extracellular water volume is governed by the influx through the skin and the bladder wall balanced by urine production and the voiding of urine stored in the bladder and, on land, by evaporative water loss (4, 23, 64, 101, 728, 1221, 1489, 1615, 1764, 1765). The studies discussed below indicate that amphibian water and salt balance, besides being submitted to moderate fluctuations, is under dynamic, temporal control governed by, for example, food intake, temperature and environmental osmotic conditions that include transitions between the aquatic and the terrestrial environment and habitats of restricted availability of free water.

Moderate fluctuations and anticipatory drinking

Table 9 indirectly indicates that the balance of cutaneous water uptake and water excretion is submitted to variations. In a direct study, Jørgensen (868) observed variations in hydration of ±5% of the body mass with empty urinary bladder in undisturbed *B. bufo* kept in a simulated terrestrial habitat at 16°C. The toads spent most of the time outside the pool of water, where they experienced evaporative water loss of ~3% of the body mass per day. Interestingly, the toads initiated cutaneous drinking in a hydrated state, that is, before EWL dehydrated the body, and generally before the urinary bladder was empty, for which the phrase *anticipatory drinking* was introduced, contrasting *emergency drinking* in dehydrated animals. The results of this and a detailed review of other studies led Jørgensen to conclude that normally undisturbed amphibians on land are well hydrated, underscoring that cutaneous drinking in terrestrial anurans is not necessarily elicited by dehydration (860, 865, 868, 869).

Food intake

Food consumption initiates drinking, and the water taken up exceeds the mass of the food eaten, which secures secretion of fluids with digestive enzymes into the gastrointestinal tract. In *B. bufo*, the cutaneous water uptake is proportional to the size of the meal and may amount to as much as 15% of the standard body mass. The spadefoot toad (*Scaphiopus*), which is active above ground a few days in a year, may consume as much as 55% of its body mass in a single feeding, which initiates cutaneous drinking in a similar way (435). Eventually, excess water is stored in the bladder, which allows the animal to remain hydrated between rainfall periods in the burrows (1587).

Aquatic versus terrestrial living

Water-acclimated amphibians exhibit high rates of glomerular filtration ensuring the cutaneous uptake of water being balanced by voiding of highly diluted urine. It has been estimated that the recirculation of bladder urine amounts to less than 3% of the cutaneous influx of water in water-acclimated toads (860). During contact with wet soil, the water uptake by the skin of the terrestrial *R. temporaria* closely balanced urine production, so that the reabsorption of bladder urine amounted to less than 6% of the cutaneous water uptake (1698). Thus, when environmental water is available, water permeability of the urinary bladder is small so that reabsorption of water from the bladder urine is of marginal importance for body water balance. In dry environments, the urine production decreases significantly because of a decreased glomerular filtration rate and increased fractional water reabsorption both in anurans (1189, 1624) and in caecilians (1765). In a comprehensive study, Jørgensen concluded that undisturbed toads only void urine exceptionally, so that the emptying of the urinary bladder "is practically exclusively by resorption through the bladder wall" (869). Thus, on land, the urine produced is stored in the bladder and recycled into the body fluids for maintaining water balance during evaporation of water into the atmosphere. This is accomplished by an increased hydrosmotic permeability of the bladder epithelium, which overcomes the higher osmolality of the bladder urine (e.g., [1765]).

A two-three-fold stimulation of the cutaneous water permeability of hydrated *B. bufo* transferred from wet to dry environment takes place within an hour. Immediately, the rate of urine production starts to decline. Both of these responses occur before body water volume decreases. It follows that they are elicited by exposure to dehydrating conditions *per se*, not in response to dehydration. The time course of the water permeability decrease following transfer of hydrated toads from dry environment to water takes two to five days. However, the urine production rose immediately after the toads were transferred to water. Taken together, the above observations indicate that functions of kidney, bladder and skin in maintaining water balance are regulated in non-dehydrated animals

(860, 869). The mechanisms that integrate rate of urine production and water permeabilities of skin and bladder wall of non-dehydrated animals are not known in detail.

Temperature and hibernation

Extracellular volume and osmolality depend on environmental temperature (617, 864, 1116, 1225, 1624). *R. catesbeiana* transferred to water at 2-5°C initially stopped urine production but not cutaneous water uptake, resulting in a body mass increase of 5-6%. After about a day, urine production commenced but at a 75% lower rate. This was due to a significantly decreased GFR from about 34 $ml \cdot kg^{-1} \cdot hr^{-1}$ to 4.5 $ml \cdot kg^{-1} \cdot hr^{-1}$ associated with low tubular reabsorption of water (1624). In the cold temperate zone, amphibians spend about half of the year hibernating, during which total body water is reversibly increased (166, 870, 1308). A study on *B. bufo* kept in 1/25 Ringer's solution showed that water uptake at 4°C was followed by a delayed cutaneous ion uptake. At the new steady state, the accumulated fluid volume amounted to 80% of the standard body mass that was partitioned both to the extracellular (70%) and intracellular space (10%). Lymph concentrations of Na^+ and Cl^- were elevated to levels above their values prior to hibernation, whereas K^+ dropped to 1.9 mM and remained low until the temperature was raised to 20°C. Thus, extracellular ion concentrations, as well, depend on environmental temperature with opposite displacements of the two alkali metal ions (870, 1308). Studies of overwintering frogs and tadpoles of the aquatic *R. muscosa* indicated similar but quantitatively smaller water and electrolyte accumulations (166). The above-mentioned acute water retention in anurans on cooling to a low temperature resembles that of dehydrated animals at room temperature. Isotonic volume expansion of the extracellular fluid prior to cooling did not prevent the water retention response, which therefore was suggested to be osmoregulatory rather than volume regulatory. The increased rate of cutaneous ion uptake seen after a few days at low temperature indicates a reaction similar to when salt depleted (863, 867).

Body water balance in habitats of restricted availability of free water

The anurans *Scaphiopus* spp., *B. viridis*, *X. laevis* and *R. cancrivora* and the urodeles *Ambystoma tigrinum* and *Batrachoseps* spp. can cope with osmolalities of the body fluids that are two to four times above the range of values of other amphibians (see Table 7), which is associated with reduced urine flow and storing of hypoosmotic bladder urine. This enables cutaneous water uptake from surroundings of poor availability of free water and recycling of water from the bladder urine into extracellular water. Plasma concentrations of Na^+, Cl^- and especially urea, but not K^+, are elevated (69, 418, 630, 782, 854, 1191-1194, 1574, 1671) by renal regulation of volume and composition of the ureteral urine (1632, 1678). Increased intracellular concentrations of Cl^-, K^+, urea and free amino acids contribute importantly to preventing fatal loss of cell water volume at osmotic equilibrium between intracellular and extracellular fluids (631, 781, 899, 2039). The tolerance to high plasma osmolalities by the above physiological acclimatizations has expanded the niche exploitations of amphibians to include deserts and aquatic environments of high salinity (67, 759, 866, 893).

Reptilia

Reptiles exist and flourish in environments ranging from completely aquatic, both freshwater and marine, to arid terrestrial. However, there is no common water and electrolyte *milieu intérieur* that applies to all orders or species, and the *milieu intérieur* in any given species is not as tightly controlled as it is in birds and mammals (175, 392). Indeed, many systematists do not believe that the reptiles really exist as a separate class, only as amniotes that are neither birds nor mammals (1248). However, I continue to treat reptiles as a single class of vertebrates, like the amphibians, birds and mammals, as I review their osmoregulation and excretion. The primary sites involved in osmoregulation are skin, kidneys (including postrenal modification of the urine in the colon, cloaca, and bladder) and salt glands.

Function and Regulation of the Physiological Mechanisms for Osmoregulation

Skin

Skin forms an important potential route for the exchange of water and, in aquatic reptiles, ions with the external environment. The appearance of reptilian skin suggests that it might be impermeable to water, as it was once thought to be. But it is not. Indeed, as shown in Table 11, it is the principal route for evaporative water loss (~70-90% of the total) in semiaquatic and terrestrial reptiles (103, 280). Even in aquatic reptiles, the integument is highly permeable to water (462, 464, 1566). The permeability of the skin to water, determined as the rate of evaporative water loss under identical conditions, decreases with increasing aridity of the habitat (also often reported as increasing resistance to water loss with increasing aridity), a pattern observed across orders and across species in a single order (Tables 1 and 2) (103, 437, 579, 1053, 1186, 1187, 1559, 1560) (see also Table 2 in Lillywhite [1053] for a more exhaustive list). This relationship has also been observed within geographically isolated populations of a single species (438). Moreover, for some species the rate of evaporative water loss across the skin has been shown to decrease when they are exposed to an arid environment (891, 952).

The permeability of the integument of aquatic species to water decreases as the salinity of their normal habitat increases and this holds across orders and across species (Table 12) (462, 464). However, a puzzling observation at odds with all

Table 11 Examples of Cutaneous Evaporative Water Loss in Living Reptiles in Dry Air

Species	Normal Habitat	Cutaneous Water Loss mg cm^{-2} day^{-1}	Percent TEWL
Crocodilia			
Caiman sclerops	Semiaquatic, freshwater	32.9±2.45 (8)	87±2.1 (8)
Testudinea			
Pseudemys scripta	Semiaquatic, freshwater	12.2±1.44 (6)	78±2.7 (8)
Terrapene Carolina	Terrestrial, mesic	5.3±0.41 (6)	76±3.4 (6)
Squamata			
Sauria			
Iguana iguana	Terrestrial, mesic	4.8±0.50 (8)	72±4.3 (8)
Saurmalus obesus	Terrestrial xeric	1.3±0.10 (6)	66±2.0 (6)

Values are means ± SE; numbers in parentheses equal number of animals; TEWL equals total evaporative water loss. Data are from Bentley and Schmidt-Nielsen (103). Table from Dantzler and Bradshaw (392) with permission.

other observations is that two species of sea snakes (*Hydrophis ornatus* and *H. inornatus*) have an integument nearly as permeable to water as that of purely freshwater snakes (Table 12). Because these two species maintain the osmotic and ionic composition of their plasma far away from that of sea water and similar to that of other sea snakes, the physiological significance of this observation is unclear (462). The skin of aquatic reptiles also has a substantial permeability to sodium, and, at least among snakes, it is significantly greater in freshwater species than in estuarine or marine species (462). The high

Table 12 Examples of Efflux and/or Influx of Water in Reptiles in Seawater

Species	Normal Habitat	Water Efflux ml $100g^{-1}$ h^{-1}	Water Influx ml $100g^{-1}$ h^{-1}
Testudinea			
Chrysemys picta	Semiaquatic, freshwater	—	0.72±0.11 (3)
Malaclemys Terrapin	Semiaquatic estuarine	0.16±0.05 (11)	0.17±0.03 (11)
Squamata			
Ophidia			
Nerodia sipedon	Semiaquatic, freshwater	1.54±0.54 (5)	1.33±1.19 (5)
Nerodia fasciata Pictiventris	Semiaquatic, freshwater	2.84±2.00 (4)	2.54±2.49 (4)
Acrochordus Granulatus	Semiaquatic, estuarine	0.49±0.04 (4)	0.47±0.19 (4)
Laticauda Laticauda	Aquatic, seawater	0.20±0.06 (4)	0.17±0.05 (4)
Hydrophis belcheri	Aquatic, seawater	0.61±0.06 (2)	0.54±0.21 (2)
Hydrophis ornatus	Aquatic, seawater	1.26±0.27 (8)	1.08±0.32 (8)
Hydrophis Inornatus	Aquatic, seawater	1.18±0.52 (4)	1.19±0.28 (4)

Values are means ± SD. Numbers in parentheses equal number of measurements. Data were determined from isotopic fluxes. Data on Testudinea are from Robinson and Dunson (1566); those on Squamata are from Dunson (462). Adapted from Dantzler and Bradshaw (392) with permission.

Table 13 Examples of Water Loss Through Shed Skin of Reptiles

Species	Normal Habitat	EWL mg cm^{-2} h^{-1} Untreated	Extracted	Lipid Content % dry weight
Ophidia				
Acrochordus Javanicus	Aquatic, freshwater	0.50±0.08 (10)	1.30±0.13 (8)	2.43
Nerodia Rhombifera	Semiaquatic, freshwater	0.41±0.13 (7)	2.62±0.50 (9)	4.30
Elaphe Obsolete	Terrestrial, mesic	0.22±0.01 (39)	2.53±0.34 (44)	5.91
Crotalus Adamanteus	Terrestrial mesic	0.22±0.07 (17)	2.59±0.33 (21)	5.89
Crotalus Viridis	Terrestrial semi-arid	0.14±0.04 (4)	2.92±0.38 (6)	7.40
Crotalus Cerastes	Terrestrial xeric	0.16±0.04 (8)	2.40±0.40 (10)	8.61
Sauria				
Iguana iguana	Terrestrial, mesic	1.16±0.09 (5)	1.98±0.12 (4)	

Values are means ± SE; numbers in parentheses equal number of measurements; EWL equals evaporative water loss. Data are from Roberts and Lillywhite (1559, 1560). Table from Dantzler and Bradshaw (392) with permission.

rate of sodium influx that this permits in freshwater species may explain their intolerance to seawater (462).

Within reptilian skin, the epidermis forms the limiting barrier for water exchange and, in aquatic species, for ion exchange with the environment. Within the epidermis, lipids form the major barrier for the diffusion of water (681, 682, 1559, 1560, 1775). Extraction of lipids from the shed skins of ophidian and saurian reptiles eliminates the barrier to evaporative water loss (Table 13), but denaturation of proteins has little effect (1056, 1559, 1560).

The lipids involved in restricting water permeation are found in multiple bilayer sheets, consisting primarily of highly saturated, unbranched, long-chain ceramides extruded by lamellar granules into the extracellular compartment of the mesos layer of the epidermis (987, 1019, 1053, 1056, 1559, 1967). In reptiles, these epidermal lipids also include the unusual glycolipids, glucosylsterol and acylglucosylsterol (3). It is not yet known whether the lipid composition differs between species from arid and mesic environments or changes with adaptation of a single species to a more arid or more mesic environment. However, Roberts and Lillywhite (1560) found that total lipid content of the ophidian epidermis increases as water permeability decreases (Table 13), and it is possible that the quantity of lipids is more important than their composition in determining water permeability.

Recently, the water channel aquaporin 3 (AQP3) has been found in mammalian epidermis (1737), although it appears to be involved in maintaining epidermal hydration, not in transepidermal water permeability. Whether an ortholog of AQP3 or any other water channel that could play a role in water permeability is present in reptilian epidermis has yet to be determined.

The way in which inorganic ions permeate the skin of aquatic reptiles is not at all clear. In general, movement of inorganic ions across lipid membranes requires protein channels or transporters, and no such channels or transporters have been described in reptilian epidermis. Extraction of lipids from shed ophidian skin markedly increases the permeability to inorganic ions (1775), as would be expected, but this does not explain how inorganic ions cross the skin when the lipids are present, especially in view of the observation that extraction of proteins alone has little or no effect on the permeability of inorganic ions (1775). In addition, Stokes and Dunson (1775) reported that there was directional asymmetry in tracer fluxes of sodium (as well as water) across shed skins that were abolished by lipid extraction. This finding is controversial and has yet to be confirmed, but it suggests that some aspect of lipid composition could actually play a role in ion permeability across the epidermis.

No information is available about any physiological regulation of ion and water movement across reptilian integument, although it is possible that regulation of vascular perfusion of the skin could influence such movements. Any effect would, of course, depend on the relationship of the vascular rate of delivery of ions and water and the permeability of the epidermis to them. No information on the regulation of vascular perfusion or its possible effects on magnitude of ion and water movements across the epidermis is available for reptiles. It should also be noted that almost all information available about ion and water exchange across the

reptilian skin has come from studies on squamates, primarily ophidians; very little is from studies on chelonians and crocodilians.

Kidneys

The kidneys play a critical role in regulating the composition of the internal environment of reptiles, as they do in other tetrapod vertebrates, by controlling the excretion of water, ions, and nitrogenous wastes. However, in all reptile species, structures distal to the kidneys (colon, cloaca or bladder) can modify the composition of the urine before it is excreted, and, in some reptile species, salt glands contribute to the excretion of ions. Nevertheless, the kidneys are quantitatively more important than any other structures in regulating the output of ions and water and, therefore, the composition of the internal environment. This regulation involves adjustments both in filtration at the renal glomerulus and in reabsorption and secretion along the renal tubules.

General form of kidneys and nephrons

The external shape of reptilian kidneys varies extensively, apparently being determined by the marked variation in body type between the different orders of the Class Reptilia. For example, the kidneys of snakes are long and thin, those of lizards are compact, somewhat triangular-shaped organs joined at the posterior end, and those of turtles are shaped by the carapace (W. H. Dantzler, personal observations) (177). However, the basic components of the nephrons, the actual functional units of the kidneys, are the same in all reptiles. Each includes a glomerulus (except for a few nephrons observed in one species of lizard [1349] and in some snakes [1515]); a short, ciliated neck segment; a proximal tubule; a short, ciliated thin intermediate segment; and a distal tubule (382). Reptilian nephrons do not have the long loops of Henle arranged parallel to collecting ducts that are found in avian and mammalian nephrons. Indeed, the gross, external shape of the kidneys constrains the gross arrangement of the nephrons within them. For example, in lizard kidneys the nephrons branch obliquely off collecting ducts and are arranged in compact bunches (1349), whereas in snake kidneys the nephrons lie side by side in neat parallel rows and attach to the major collecting ducts at roughly right angles (382). Despite these differences in gross nephron arrangement in the kidneys of the different orders of living reptiles, in all cases examined, the nephrons are arranged so that the beginning of the distal tubule is closely apposed to the vascular pole, apparently the afferent arteriole, of its own glomerulus (S. D. Yokota, R. A. Wideman, and W. H. Dantzler, unpubl. observ.) (810, 1349). Finally, it is important to note that in all reptiles studied, the capillary network surrounding the renal tubules receives blood not only from the glomerular efferent arteriole but also from a renal portal system supplying venous blood from the posterior regions of the body.

Glomerular function

The initial process in the formation of urine is the delivery of water and solutes from the blood to the lumen of the proximal renal tubules. In glomerular nephrons (almost all reptilian nephrons), this involves the ultrafiltration of plasma water and solutes across the glomerular capillary walls, a process first documented in reptiles in 1933 by Bordley and Richards (152). They collected fluid from Bowman's space around the glomerular capillaries and found it to be protein-free (within the limits of their measurements) and to contain small solutes in the same concentrations as in the plasma. The composition of the filtrate determines the initial composition and the rate of formation of the filtrate determines the initial volume flow rate of the tubular fluid. The production of this ultrafiltrate by arterial hydrostatic pressure maintained for other purposes is an efficient way to excrete large volumes of fluid without expending additional energy specifically for this purpose. Moreover, because the rate of formation of glomerular filtrate can be varied readily (see below), it can be an important determinant of the volume of urine excreted. Those few aglomerular nephrons observed in some reptilian species, if they function at all, must do so by secreting ions and water across the tubule epithelium into the lumen (see below), but these nephrons are clearly not of much significance in the formation of reptilian urine.

The rate of ultrafiltration at the glomerulus of a single nephron (SNGFR) is the product of the ultrafiltration coefficient (K_f) and the net ultrafiltration pressure (PUF):

$$\text{SNGFR} = K_f[(P_{GC} - P_{BS}) - \pi_{GC}]$$

Within the brackets, P_{GC} is the outwardly directed hydrostatic pressure in the glomerular capillaries, P_{BS} is the inwardly directed hydrostatic pressure in Bowman's space, and π_{GC} is the colloid osmotic pressure in the capillaries, which opposes filtration. The sum of these pressures is the PUF. Almost no protein is normally filtered and, therefore, essentially no colloid osmotic pressure exists outside the capillaries to favor filtration. Yokota, working with anesthetized garter snakes (*Thamnophis* spp.), measured (in mm Hg) a mean arterial pressure of 38, a mean P_{GC} of 22, a mean P_{BS} of 2, and a mean π_{GC} in the afferent arteriole of 17 (S. D. Yokota, pers. comm.). These data, the only such measurements available for reptiles, yield a PUF of 3 mmHg at the afferent end of the glomerular capillary network. Because Yokota made the P_{GC} measurements randomly in the glomerular capillaries, it seems probable that, as in mammals, P_{GC} falls only very slightly along the length of the glomerular capillary network. This nearly constant P_{GC} is maintained by resistances in the afferent and efferent arterioles. As filtration occurs along the length of the capillary network, π_{GC} rises. Whether or not it rises sufficiently in garter snakes to equal P_{GC}, thereby causing filtration to cease before the efferent end of the capillary network, is unknown. However, because the value of PUF at the afferent end of the glomerular capillaries is so low, it

seems likely that this does occur in garter snakes and possibly in other reptiles. If this filtration equilibrium is normally reached before the efferent end of the capillary network (i.e., not all of the network is used for filtration), the SNGFR will be particularly sensitive to changes in renal plasma flow.

As indicated above, SNGFR is a function, not only of PUF, but also of K_f, which itself is a function of the surface area of the capillaries available for filtration (A) and the aqueous permeability of the capillary wall (hydraulic conductivity or L_p). A measurement of either A or L_p would permit determination of the other from that value, the PUF, and the SNGFR. However, at present, it is not possible to measure either A or L_p accurately for any species. Therefore, it is only possible to calculate their product, K_f, from the PUF and the SNGFR. In the case of garter snakes, the only reptile for which data are available, a PUF of 3 mmHg and SNGFRs ranging from 0.6 to 5 nl/min (152) (S. D. Yokota and W. H. Dantzler, unpubl. observ.) yield K_f values of 0.2 to1.7 nl/min/mmHg (392). The larger value is only about one-third that in Munich-Wistar rats (196, 411), less than one fourth that in Congo eels (*Amphiuma means*) (1432), but about equal that in river lampreys (*Lampetra fluviatilis*) (1216).

The rate of formation of glomerular filtrate for the whole kidney (GFR) is the sum of all the SNGFRs for that kidney. However, it is not possible to determine experimentally all the SNGFRs and sum them in the kidneys of living animals. Fortunately, it is possible to determine the whole-kidney GFRs (for both kidneys combined) by clearance methods in living animals.

A number of whole-kidney GFR measurements have been made in some members of all four extant orders of living reptiles during acute changes in hydration or during a water load (usually hypoosmotic glucose solution) or a salt load (usually 1 mol/l hyperosmotic sodium chloride) (Table 14) (see also table 8.1 in [189]). In general, whole-kidney GFRs tend to increase with a water load and decrease with dehydration or a salt load, but there is considerable variation among species, particularly in response to a salt load, where an increase in GFR frequently occurs (Table 14). This latter variation in response to a salt load may reflect the fact that a hyperosmotic salt load in a well-hydrated animal, although given to mimic dehydration, would initially tend to lead to plasma expansion, increased renal blood flow, and increased GFR. Only with prolonged infusion, which was not generally performed, would a salt load in a well-hydrated animal lead to water and volume depletion, an increase in plasma osmolality and a decrease in GFR. Also, lingual salt glands (see below) in crocodiles and wholly aquatic sea snakes may have removed sodium chloride rapidly enough to make the salt load equivalent to an isosmotic plasma expansion or even a hypoosmotic water load, thereby leading to an increase in GFR. In some studies (Table 14), renal function was observed to cease completely when plasma osmolality increased sufficiently, thereby eliminating renal water excretion at the expense of retaining salts and metabolic wastes. However, this occurred at much higher plasma osmolality in the desert tortoise than in semiaquatic or mesic species, suggesting that these desert animals tolerate much wider swings in plasma osmolality than other species (395, 1286). In summary, it appears that reptiles, in contrast to mammals and despite some variation among species, show marked increases or decreases in whole-kidney GFR with acute physiological changes in hydration, suggesting that changes in GFR play an important physiological role in regulating the volume and composition of the final urine (see below).

These changes in whole-kidney GFR appear to result primarily from changes in the number of filtering nephrons, although variation in the filtration rate of those nephrons filtering probably also plays some role (381, 395, 1623, 2056). The concept that a change in the number of filtering nephrons is the primary process involved in a change in whole-kidney GFR in reptiles is supported indirectly in those species studied by histological evidence that the ratio of open to collapsed proximal tubule lumina correlates roughly with the whole-kidney GFR (1623) and by data showing that the maximum rate of tubular transport of a secreted substance (*para*-aminohippurate, PAH) varies directly with whole-kidney GFR (381, 395). Of greater significance, direct quantitative measurements of blood flow rates in single glomeruli of garter snakes (*Thamnophis sirtalis*) confirm the presence of intermittent blood flow and, presumably, of intermittent filtration, and also show that the fraction of glomeruli with intermittent blood flow increases with increasing plasma osmolality (2056). Because plasma osmolality also increases with dehydration, these correlations strongly support the concept that the decreases in whole-kidney GFR observed with dehydration are the result of decreases in the number of filtering nephrons. Because the release of arginine vasotocin (AVT), the antidiuretic hormone in reptiles, is also stimulated by increases in plasma osmolality (178, 392), this further correlation strongly suggests that AVT plays a role in producing glomerular intermittency during dehydration, at least in ophidian reptiles. In further support of this concept, intravenous infusions of AVT in garter snakes cause blood flow in individual glomeruli to cease (Fig. 29) (2056) just as they produce decreases in whole-kidney GFR in water snakes (*Natrix sipedon*) (381). Varying the whole-kidney GFR by varying the number of nephrons filtering seems reasonable in animals whose nephrons are not structured to function in concert to produce urine hyperosmotic to plasma. Moreover, as noted above, reptiles have a renal portal system, which can supply the cells of non-filtering nephrons in the absence of a postglomerular arterial blood supply.

Mechanistically, decreases in blood flow in individual glomeruli in garter snakes apparently result from constrictions of the afferent glomerular arteriole in response to AVT (either endogenous or exogenous) interacting with V_1-type receptors (Fig. 29) (2056). When the diameter of the afferent arteriole becomes sufficiently small, the rigid, nucleated red blood cells cannot pass, occluding the vessel and causing cessation of flow and filtration (Fig. 29) (2056). However, filtration at an individual glomerulus may actually cease

Table 14 Changes in Whole-Kidney GFR

Species and Habitat	Condition	GFR ml kg^{-1} h^{-1}	References
Crocodilia			
Crocodylus johnsoni (freshwater, semiaquatic)	Control	6.0±1.5	(1623)
	Dehydration	1.9±0.2	
	Water load	3.3±1.1	
Crocodylus porosus (salt water, semiaquatic)	Control	1.5±0.2	
	Salt load	2.8±0.9	
	Water load	18.8±2.3	
Testudinea			
Gopherus agassizii Desert tortoise (arid, terrestrial)	Control	4.7±0.60	(395)
	Salt load	2.9±0.91	
	No urine flow when plasma osmolality ↑ 100 mOsm		
	Water load	15.1±.64	
Pseudemys scripta Freshwater turtle (fresh water, semiaquatic)	Control	4.7±0.69	
	Salt load	2.8±0.90	
	No urine flow when plasma osmolality ↑ 20 mOsm		
	Water load	10.3±2.00	
Squamata			
Sauria			
Tiliqua scincoides Blue-tongued lizard (terrestrial)	Control	15.9±1.0	(1623)
	Dehydration	0.7	
	Salt load	14.5±0.5	
	Water load	24.5±2.0	
Phrynosoma cornutum Horned lizard (arid, terrestrial)	Control	3.5±0.323	(1562)
	Dehydration	2.1±0.20	
	Salt load	1.7±0.40	
	Water load	5.5±0.54	
Hemidactylus sp. Puerto Rican gecko (moist, terrestrial)	Control	10.4±0.77	(1562)
	Dehydration	3.3±0.37	
	Salt load	11.0±2.18	
	Water load	24.3±1.67	
Ophidia			
Nerodia sipedon Freshwater snake (fresh water, semiaquatic)	Salt load	13.1±1.26	(379, 381)
	No urine flow when plasma osmolality ↑ 50 mOsm		
	Water load	22.8±1.75	
Aipysurus laevis Olive sea snake (salt water, aquatic)	Control	0.78 (0.49-2.78)	(226)
	Salt load	2.24 (1.41-6.42)	
	Chronic intraperitoneal seawater load	7.05±(6.26-7.83)	
	Water load	0.17±(0.03-0.35)	
	Chronic intraperitoneal water load	5.67±(4.40-6.20)	
Rhynchocephalia			
Sphenodon punctatum (moist terrestrial)	Control	3.9	(1627)
	Dehydration	3.4	
	Water load	4.8	

Values are means or means ± SE except for sea snakes where, because data did not show a normal distribution, the values are given as medians and interquartile ranges. All means with SE and medians are for 4 or more values. Modified from Dantzler and Bradshaw (392) with permission.

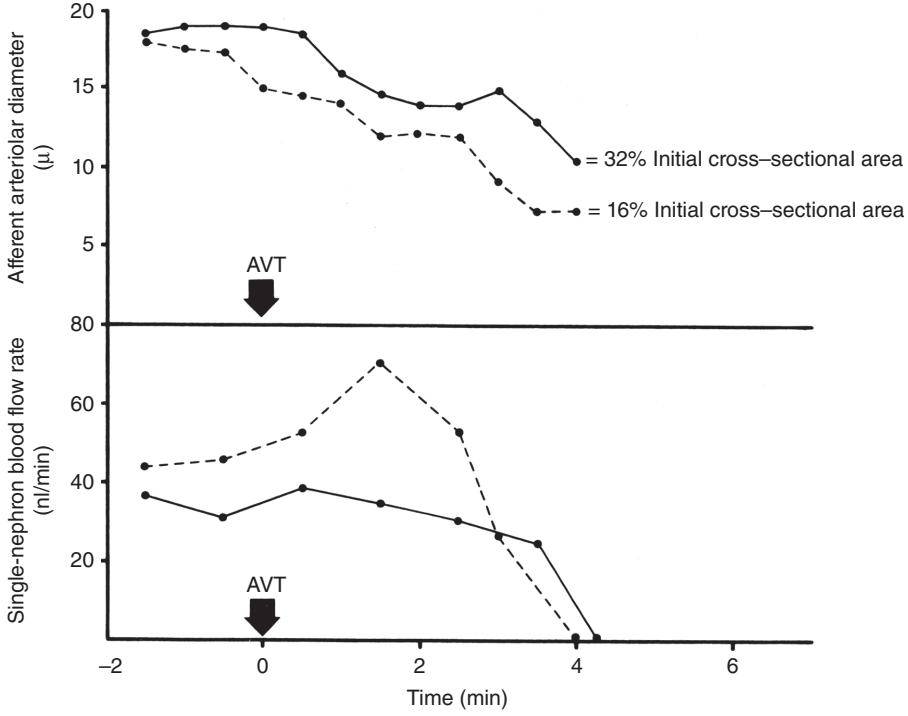

Figure 29 Simultaneous relationship of afferent arteriolar diameter and blood flow for two representative nephrons in snake (*Thamnophis sirtalis*) kidney during continuous infusion of arginine vasotocin (AVT). Arrows mark the start of AVT infusion at a rate of 17 pg 100 g^{-1} min^{-1}. Reproduced from Yokota and Dantzler (2056) with permission.

before the blood flow ceases or may not occur at all during periods of continuous low blood flow. This could occur if the outwardly directed hydrostatic pressure in the glomerular capillaries decreased sufficiently to equal the sum of the opposing colloid osmotic pressure of the plasma proteins and the hydrostatic pressure in Bowman's space (i.e., PUF fell to zero). This seems likely in garter snakes given their low PUF and quite likely in other reptiles as well, although PUF has not been determined in any other species.

In addition to AVT, the hormone prolactin may play a role in regulation of glomerular blood flow and, thus, filtration. In some freshwater turtle species (definitely in *Chrysemys picta* and possibly in *Pseudemys scripta*), prolactin administration increases whole-kidney GFR in intact animals and reverses the decrease in whole-kidney GFR observed in hypophysectomized ones, possibly by relaxing the afferent arteriole (197). It is also possible that there is some neural control of glomerular blood flow in some ophidian reptiles. Inhibitor studies in sea snakes (*Aipysaurus laevis*) and garter snakes suggest that α-adrenergic agonists, whose release is stimulated by high plasma potassium concentrations, may regulate GFR by constricting the afferent arteriole (S. Benyajati, S. D. Yokota, and W. H. Dantzler, unpubl. observ.) (108, 2055). However, more direct studies are required to determine the exact roles, if any, of both prolactin and α-adrenergic nerves in regulation of glomerular blood flow and filtration.

Autoregulation, the process in mammals whereby mechanisms intrinsic to the kidney maintain both renal blood flow and GFR relatively stable independent of changes in mean arterial blood pressure, has yet to be examined in reptiles. However, it seems unlikely to be significant in view of the highly variable mean arterial pressure, variable SNGFRs, intermittent nephron function and lack of a macula densa (382, 1316, 1726) (despite apposition of the early distal tubule to the vascular pole of its own glomerulus). However, the study of Yokota and Dantzler (2056) suggests individual glomeruli in snake kidneys may always have either high or low blood flow and, thus, high or low SNGFR, and this may be regulated in some way.

Tubule function

The renal tubules modify the initial glomerular filtrate by the processes of reabsorption and secretion, thereby determining the final volume, ionic composition and osmolality of the ureteral urine. The following discussion is limited to tubule transport of sodium, potassium, water and the primary excretory end products of nitrogen metabolism.

Inorganic ion transport *Sodium*. Although neither the concentration of sodium in the extracellular fluid nor the extracellular fluid volume itself is maintained as constant in reptiles as in mammals (173), maintaining total body sodium within some broad general range, primarily by regulating excretion, is still critically important for normal physiological function.

Most of this regulation occurs via renal tubule transport of sodium.

Although clearance studies based on collections of ureteral urine show that reptilian renal tubules may reabsorb 50% to 98% of the filtered sodium, depending on the species and the physiological circumstances of the experiments, on average at least 10% of the filtered sodium escapes reabsorption (382, 392, 1776). This is far more than in mammals where less than 1% normally escapes reabsorption. However, further regulation of sodium excretion in reptiles can occur in the bladder, cloaca or colon (see below). Moreover, the clearance studies in reptiles do not include any filtered sodium that is contained in urate precipitates in uricotelic reptiles (see below) and, therefore, may actually overestimate the fraction of filtered sodium reabsorbed by the renal tubules. In addition, clearance studies only indicate net sodium transport, not whether both secretion and reabsorption of sodium occur.

In reptiles, in contrast to mammals, the proximal tubule is not necessarily the site where the largest fraction of filtered sodium is reabsorbed. *In vitro* microperfusion studies of snake (*Thamnophis* spp.) renal tubules and *in vivo* micropuncture studies of lizard (*Sceloporus cyanogenys*) renal tubules suggest that only about 35-45% of filtered sodium is reabsorbed along the proximal tubules whereas about 50-70% is reabsorbed along the distal portions of the nephrons (382, 390, 1776). The fraction reabsorbed in a given segment reflects the relationship among the amount of filtered sodium reaching that segment, the length of the segment and rate of reabsorption per unit length. The few data available suggest that the rate of sodium reabsorption is less than or equal to that of mammals along the proximal tubule and equal to that of mammals along the distal tubules (382, 390, 1776).

Essentially no direct information is available about the mechanism of sodium reabsorption in reptilian proximal tubules, and the steps in the transport process must be inferred from a few measurements on garter snake (*Thamnophis* spp.) tubules and analogy to other vertebrates. A very small, lumen-negative transepithelial potential (ca. −0.50 mV) in proximal tubules indicates that transepithelial sodium transport is against an electrochemical gradient (388). Moreover, because fractional reabsorption of chloride is the same as that of sodium in the proximal tubule (390), it appears that transepithelial transport of sodium chloride involves active transport of sodium and passive transport of chloride. This may be true for other reptile species as well.

A basolateral membrane potential of about −60 mV (924) in garter snake proximal tubules indicates that, as in other vertebrates, sodium can enter the cells from the lumen down an electrochemical gradient. Also, because a functional sodium-hydrogen exchanger has been demonstrated in brush border membrane vesicles from these kidneys (396), it seems very likely that, as in mammals, luminal sodium entry primarily involves such exchange. This possibility is indirectly supported by observations that administration of a carbonic anhydrase inhibitor to water snakes (*Nerodia sipedon*) results in ureteral urine that is alkaline and contains increased amounts of sodium and potassium (379). In addition to entering the cells across the luminal membrane by exchange for hydrogen, some sodium almost certainly enters coupled to the entry of glucose, amino acids, phosphate and other molecules. As in other vertebrates studied, transport of sodium out of the cells against an electrochemical gradient at the basolateral membrane almost certainly involves Na^+-K^+-ATPase, which is present in the basolateral membrane of proximal tubules in snakes (107, 380) and most likely other reptiles.

In vitro microperfusion studies of snake (*Thamnophis*) distal tubules (117, 119, 122) provide insights into a possible intrinsic control mechanism for regulating sodium reabsorption in this tubule segment. These studies reveal a substantial lumen-negative transepithelial potential (ca. −35 mV), which is inhibited by ouabain on the basolateral side or amiloride on the luminal side. The calculated short-circuit current (considered to represent sodium reabsorption) and the transepithelial potential, while dependent on the presence of sodium in the lumen, both decay rapidly when the luminal sodium concentration exceeds 30 mM. The decays in transepithelial potential and short-circuit current appear to represent an increase in resistance to sodium transport through the active transport pathway. Under these conditions the rate of active sodium extrusion across the basolateral membrane is not great enough to keep up with the rate of passive sodium entry across the luminal membrane, and the cells swell. This intrinsic response to a large sodium load in the distal tubules may help prevent the reabsorption of too much sodium at a time when there is a need to excrete excess sodium. Also, a transport system poised to work effectively only at low luminal sodium concentrations will enhance dilution of tubule fluid even when the luminal sodium concentration is already low and thus enhance the excretion of excess water (see below). Whether such intrinsic controls are available in the distal tubules of other reptiles is unknown.

Hormonal regulation of renal tubule transport of sodium is far from clear. In clearance studies, administration of physiological doses of reptilian antidiuretic hormone, arginine vasotocin (AVT), increases fractional reabsorption of sodium in a number of species, but not all (392). In fact, in one chelonian species (*Chrysemys picta belli*), administration of AVT actually decreased fractional sodium reabsorption (231). Even if AVT tends to stimulate sodium reabsorption in most reptile species, the tubule site of such stimulation is completely unknown.

Although aldosterone and corticosterone are the major secretory products of the reptilian adrenals (1600, 1913), their action on renal tubule sodium transport—in fact, on kidney function in general—is not well understood and appears to vary among species, even of the same order (392). For example, as in mammals, aldosterone appears to stimulate tubule sodium reabsorption in the freshwater snake *Natrix cyclopion* (1026) and the lizard *Varanus gouldii* (173, 1537), but it may have the opposite effect in the lizards *Ctenophorus ornatus* and *Dipsosaurus dorsalis* (174, 179, 268). Moreover, even when aldosterone appears to stimulate tubule sodium

reabsorption, the tubule site of such stimulation is unknown. Corticosterone also has variable effects on different lizard species (392). Clearly, understanding hormonal regulation of sodium transport in reptiles requires much more study.

Potassium. Potassium, the major cation of the intracellular fluids, although particularly critical for the function of excitable cells, is not maintained as constant in reptiles as in mammals. However, there is still considerable regulation of the total quantity in reptiles, largely by renal and extrarenal excretion. Renal clearance studies in numerous species (392) and renal micropuncture studies in one species (blue spiny lizard, *Sceloporus cyanogenys*) (1776) indicate that either net tubule secretion or net tubule reabsorption may occur. Although the magnitude and sites of tubule potassium transport are not well described for most reptiles, the micropuncture study on *Sceloporus cyanogenys* (1776) suggests that about 25-35% of filtered potassium may regularly be reabsorbed along the proximal tubule under normal circumstances. Along the distal tubules, transport may vary from net reabsorption of 20% of the filtered load to net secretion of as much as 180% of the filtered load, most likely in response to varying needs to retain or excrete potassium. This pattern in *Sceloporus* distal tubules is similar to that observed in mammals and those other non-mammalian vertebrates that have been studied (382). However, the exact distal tubule sites, cell types, mechanisms or regulation (hormonal or otherwise) involved in transepithelial tubule transport of potassium in *Sceloporus* are unknown. Some additional potassium can be reabsorbed in the collecting ducts in *Sceloporus*, but this does not appear to be very significant (1776). Finally, as a cautionary note about the clearance and micropuncture studies, it is important to recognize that in uricotelic reptiles, some potassium in the tubule fluid may be combined with urate precipitates (see below) (387), and therefore, measurements involving only the aqueous phase of the urine may not give an accurate picture of the magnitude or even the direction of net tubule transport (387).

Water Transport *Water reabsorption*. Water reabsorption by the renal tubules plays a critical role in the overall water balance of reptiles. Sometimes it occurs at the same rate as solute reabsorption (as isosmotic fluid) and sometimes it lags behind, depending on the need to conserve or excrete water. However, in reptiles, total water reabsorption by the renal tubules, measured as a fraction of the filtered fluid, never equals, much less exceeds, 99%, even during dehydration, as it does in mammals (189, 383). Moreover, microperfusion, micropuncture and clearance studies indicate that only about 30-50% of the filtered fluid is reabsorbed along the proximal tubules in ophidian, saurian, chelonian and crocodilian reptiles (390, 395, 1629, 1776). This is distinctly below the minimum of at least two-thirds of the filtered fluid normally reabsorbed along the proximal tubules of mammals (188). Instead, perhaps as much as 45% of the filtered fluid can be reabsorbed along the distal tubules and collecting ducts, the exact amount often depending on the need to conserve water and the action of antidiuretic hormone (188, 392). In considering the overall fluid balance of reptiles, however, it must be remembered that substantial amounts of water can be reabsorbed in the colon, cloaca or bladder, depending on the needs of the animal (see below).

The mechanism by which filtered fluid is reabsorbed in the proximal tubules of reptiles is not understood, although microperfusion studies in snakes (*Thamnophis* spp.) and micropuncture studies in lizards (*Sceloporus cyanogenys*) indicate that the fluid in the proximal tubule remains isosmotic with the plasma during reabsorption (390, 1776). However, although these studies indicate that sodium and water can be reabsorbed at osmotically equivalent rates, they do not indicate that fluid reabsorption always depends on sodium reabsorption. Indeed, in snake (*Thamnophis* spp.) proximal tubules perfused *in vitro*, isosmotic fluid reabsorption can occur when lithium replaces sodium in the lumen or when some other substance (e.g., choline, tetramethylammonium, methylsulfate, sucrose) replaces sodium, chloride or both in the lumen and the peritubular fluid simultaneously (390). Also, reabsorption in these studies is not influenced by the buffer used and, even when sodium is present, inhibition of Na^+-K^+-ATPase does not disrupt reabsorption (390, 391). Also, when sodium is present, about 25% of the net fluid reabsorption in the proximal tubule depends on colloid in the peritubular fluid (390). Increases in cell membrane area, which occur within minutes following simultaneous substitutions for sodium in the luminal and peritubular fluid, suggest that under these circumstances, colloid osmotic pressure in the peritubular fluid or perhaps some small, previously unimportant driving force could account for maintaining reabsorption at the control level (394). However, the exact mechanism involved in fluid reabsorption in these proximal tubules is not yet understood and offers an excellent opportunity for studies on what may be a unique epithelial transport process.

Water secretion. Clearance studies in sea snakes (*Aipysurus laevis*) suggest that net secretion of fluid at very low levels may sometimes occur when whole-kidney GFR is very low (2055). The tubule site of this fluid secretion and the mechanism involved in it are unknown, but, as in fish proximal tubules and mammalian collecting ducts (1926), the process appears to be dependent on the secretion of sodium and chloride (2055). Whatever the mechanism, fluid secretion certainly would appear to be necessary for any aglomerular nephrons in reptiles to actually function as excretory organs.

Dilution and concentration. In addition to varying GFR in response to changes in hydration (see above), many reptiles—but not all—vary the osmolality of ureteral urine from isosmotic to the plasma to significantly hypoosmotic to the plasma in response to changes in hydration (Table 15). These variations in osmolality of the ureteral urine, which reflect variations that actually occur along the renal tubules, are the principle means by which the kidneys alter the amount of solute-free water delivered to the colon, cloaca or bladder (see below). Production of tubule fluid hypoosmotic to the plasma from the isosmotic glomerular filtrate requires reabsorption of solutes,

Table 15 Examples of Range of Osmolal Urine-to-Plasma Ratios (U/P)

	Osmolal U/P (approximate maximum range)	References
Crcodilia		
Crocodile *Crocodylus acutus* (freshwater and marine, semiaquatic)	0.55-0.95	(1629)
Testudinea		
Desert tortoise *Gopherus agassizii* (arid, terrestrial)	0.3-0.7	(395)
Freshwater turtle *Pseudemys scripta* (freshwater, semiaquatic)	0.3-1.0	(395)
Squamata		
Sauria		
Horned lizard *Phrynosoma cornutum* (arid, terrestrial)	0.8-1.0	(1562)
Blue spiny lizard *Sceloporus cyanogenys* (arid, terrestrial)	0.3-0.7	(1776)
Sand goanna *Varanus gouldii* (arid, semiaquatic)	0.4-1.0	(178)
Ophidia		
Bull snake *Pituophis melanoleucus* (arid, terrestrial)	0.5-1.0	(959)
Freshwater snake *Nerodia sipedon* (freshwater, semiaquatic)	0.1-1.0	(381)
Olive sea snake *Aipysurus laevis* (marine, aquatic)	0.8-1.2	(2055)

The values are from measurements on ureteral urine. Modified from Dantzler (382) with permission.

primarily sodium and chloride, without accompanying water somewhere along the nephrons. The tubule sites of this dilution process in reptiles have generally not been well defined. However, micropuncture studies on a xerophilic lizard (*Sceloporus cyanogenous*) (1776) indicate that dilution can occur at least by the early distal tubule and that it can continue throughout the collecting duct. Preliminary microperfusions of snake (*Thamnophis* spp.) renal tubules suggest that dilution may occur in the thin intermediate segment between the proximal and distal tubules (S. D. Yokota and W. H. Dantzler, unpubl. observations), and, as noted above, the distal tubules of these animals may be specialized to permit additional dilution by sodium reabsorption from tubule fluid that is already highly dilute (117, 119, 122).

The degree to which the osmolality of the tubule fluid can be reduced relative to the plasma depends, not just on the magnitude of solute reabsorption, but also on the degree to which the tubule epithelium remains impermeable to water. Freshwater reptilian species with a major need to excrete excess water generally produce the most dilute tubule fluid (about one-tenth the osmolality of the plasma) (Table 15). Increases in ureteral urine osmolality from hypoosmotic toward isosmotic then reflect increases in the permeability of the tubules to water, apparently as a result of the interaction of AVT with V_2-type receptors in the tubules. Clearance studies support this regulatory role of AVT in freshwater snakes (*Nerodia = Natrix sipedon*) (381), freshwater turtles (*Chrysemys picta belli* and *Pseudemys scripta*) (231, 395) and arid-living lizards (*Ctenophorus ornatus* and *Varanus gouldii*) (176, 178). However, not all species show a wide range in variation in osmolality of the ureteral urine (Table 15). For example, a few species (horned lizard, *Phrynosoma cornutum*; olive sea snake, *Aipysurus laevis*) always produce ureteral urine close to the osmolality of the plasma, whereas others (desert tortoise, *Gopherus agassizii*; blue spiny lizard, *Sceloporus cyanogenys*) always produce ureteral urine hypoosmotic to the plasma. Indeed, the micropuncture studies on *Sceloporus cyanogenys* show no change in dilution along the renal tubules in response to the administration of AVT. Ureteral urine nearly isosmotic to the plasma always produced by horned lizards and olive sea snakes (Table 15) appears appropriate for these species, which rarely have excess water to excrete. However, ureteral urine being always hypoosmotic to the plasma produced by desert tortoises and blue spiny lizards (Table 15) appears inappropriate, for these species also rarely have excess water to excrete. Further modification of the urine in these latter two species clearly occurs in the bladder or cloaca (see below).

Table 16 Approximate Percentages of Total Urinary Nitrogen as Urates, Urea and Ammonia

	Percent of total urinary nitrogen as			
	Urates	Urea	Ammonia	References
Crocodilia	70	0-5	25	(917)
Testudinea				
Wholly aquatic	5	20-25	20-25	(1259)
Semiaquatic	5	40-60	6-15	(82)
Wholly terrestrial				
Mesic environment	7	30	6	
Xeric environment	50-60	10-20	5	
Desert tortoise *Gopherus agassizii*	20-25	15-50	3-8	(395)
Freshwater turtle *Pseudemys scripta*	1-24	45-95	4-44	
Squamata				
Sauria	90	0-8	insignificant to ? highly significant	(382)
Ophidia	98	0-2	insignificant to ? highly significant	
Rhynchocephalia				
Sphenodon punctatum	65-80	10-28	3-4	(751)

Modified from Dantzler (382) with permission.

Reptiles are not capable of producing urine sufficiently more concentrated than the plasma to be physiologically significant in the conservation of water. However, a few species (marine snake, *Aipysurus laevis*; lizard, *Amphibolurus maculosus*; sea turtle, *Chelonia mydas*) can produce urine slightly hyperosmotic to the plasma (maximum osmolal urine-to-plasma ratio is about 1.2-1.3) (194, 1479, 2055). In the studies on *Chelonia mydas* and *Amphibolurus maculosus*, urine was not collected directly from the ureters, and thus the slightly hyperosmotic urine obtained may actually reflect modification of ureteral urine in the bladder or cloaca. In the case of *Aipysurus laevis*, the slightly hyperosmotic urine may involve tubule secretion of solutes (e.g., sodium, potassium, magnesium or ammonium) into a very small volume of tubule fluid (2055). Although secretion of ions in these animals may be important for their excretion, the production of urine slightly hyperosomotic to the plasma cannot be of physiological significance because the plasma osmolality is far below that of sea water.

Excretory End Products of Nitrogen Metabolism: Ammonia, Urea and Uric Acid

Ammonia. Ammonia is both highly soluble and highly toxic. Thus, it becomes the primary excretory end product of nitrogen metabolism only in completely aquatic vertebrate species, where it can rapidly be carried away (189). In most vertebrate species, its primary role involves renal excretion of acid and maintenance of acid-base balance. At present, there is no information on the role of ammonia excretion in acid-base balance in reptiles. Moreover, ammonia has not been found to be the primary renal means of excreting nitrogen even in completely aquatic reptile species (189). Nevertheless, ammonia is excreted as an important end product of nitrogen metabolism in many reptile species (Table 16) (189). For example, in crocodilians such as *Alligator mississippiensis*, about 25% of the total nitrogen excreted is in the form of ammonia and about 75% is in the form of urates (Table 16) (387, 917). Moreover, in alligators on a standard meat diet, the absolute amount of ammonia excreted is greater than that for any other vertebrate studied (343, 926). Much nitrogen is also excreted as ammonia in semiaquatic and aquatic chelonians (e.g., freshwater turtle, *Pseudemys scripta*) (Table 16) (387, 395). In these animals, nitrogen excretion is frequently distributed about equally between ammonia and urea but there is much variation and urea tends to predominate (Table 16) (395).

No one has measured any significant amount of ammonia in the systemic blood of reptiles. Therefore, the ammonia present in the urine cannot come from filtration and must be produced by the renal tubules. The exact tubule site of such ammonia production is unknown, but it is generally assumed

to involve primarily the proximal tubule, as it does in mammals. Although the mechanism of ammonia production is not known in detail, Lemieux et al. (1031), studying alligator renal tubules *in vitro*, found evidence for its production from both glutamine and alanine probably by the same enzymatic deamination steps as in mammals. Interestingly, when alligators are dehydrated, renal ammonia production decreases and more nitrogen is excreted in the urine in the form of urates (926). King and Goldstein (926) suggest that during dehydration, decreased renal blood flow could decrease delivery of glutamine and alanine to the kidneys, and that accumulation of ammonia in renal tissue could drive the reversible deamination reactions in the direction of amino acid formation, but these hypotheses have not been examined directly.

No experimental data are available on the mechanism by which ammonia produced in the renal tubule cells is secreted into the tubule lumen in reptiles. In those species in which the pH of the lumen is below that of the cells, secretion could occur by non-ionic diffusion of free ammonia (NH_3) across the luminal cell membrane followed by trapping of ammonium ion (NH_4^+) in the lumen. However, carrier-mediated secretion of NH_4^+ would certainly need to occur in alligators in which the urine and, therefore, the tubule fluid are highly alkaline with much bicarbonate (341, 342). This might involve the substitution of NH_4^+ for H^+ on a Na^+/H^+ exchanger on the tubule luminal membrane.

Urea. Urea appears to be the primary form of nitrogen excretion only in some chelonian species from aquatic, semiaquatic and mesic habitats (Table 16). However, it is a highly important form of nitrogen excretion even in chelonian reptiles from arid terrestrial environments and also in the one extant rhynchocephalian species (*Sphenodon punctatum*) (Table 16). Urea is excreted primarily by filtration and tubular reabsorption, with the latter increasing with dehydration and decreasing with hydration. Indeed, net tubular secretion is observed in a few lizard species (*Lacerta viridis* and *Sceloporus cyanogenys*) and *Sphenodon* during extreme diuresis with hydration (382, 1431, 1627). The tubule sites and mechanism of tubule transport have not been examined, although both reabsorption and secretion almost certainly involve some form of carrier-mediated process.

Urate. Urate is the primary urinary form in which excess nitrogen is eliminated in all reptile species studied except for some aquatic, semiaquatic and mesic chelonian species (Table 16). The low solubility of both uric acid itself (0.384 mmol/l) and its salts (e.g., 6.76 and 12.06 mmol/l, for sodium urate and potassium urate, respectively) (674) mean that almost no uric acid and only modest amounts of urate salts can actually be dissolved in the aqueous phase of the urine. The primary cation in these urate salts that are dissolved, usually either sodium or potassium, is determined by whether the animal is a carnivore or herbivore. However, in alligators, which excrete large amounts of ammonium in an alkaline urine, ammonium urate (solubility: 3.21 mmol/l) may be the dominant salt (341, 342). In some chelonian species the concentrations of urate salts excreted may be sufficiently low that they all are in true aqueous solution (395). However, in many other reptilian species the concentrations of urates are too high to exist solely in simple solutions. Instead, the aqueous phase of the urine probably contains most urates in the form of relatively small lyophobic colloid particles and much larger lyophilic colloids (1232, 1470).

Most urate excreted by reptiles actually is in the form of precipitates, not only in the bladder or cloaca, where precipitation might be expected to occur, but also in the collecting ducts and ureters (1237) (W. H. Dantzler, unpublished observations). In the collecting ducts and ureters, these precipitates are in the form of small, smooth spheres of unknown chemical structure, which should pass along the these tubular structures without causing damage; in the bladder or cloaca of the same species, they are in the form of sharp-edged crystals, which may have formed only in those regions, as well as the small spheres (1237). These precipitates, whatever their exact chemical structure, contain significant amounts of inorganic cations, largely sodium or potassium, but sometimes calcium or magnesium (387, 1234). In alligators, they may contain ammonium (341, 342). The exact cations in the precipitates depend on the acid-base status of the animal as well as on dietary intake (387, 1234). In any case, the cations combined with these precipitates are excreted without water and do not contribute to the osmolality of the urine. Therefore, the osmolality of the urine in reptiles does not reflect the ability of these animals to excrete inorganic ions. In addition, in the colon, cloaca or bladder, less complex precipitates may form and urate salts may be converted to uric acid, particularly during the acidification observed in bladders of some turtles and cloacae of some lizards (640, 1236, 1760). This process can free up cations for further reabsorption, if required.

In all reptiles studied, the urate in the plasma is freely filtered (152) and net secreted by the renal tubules (382). Almost all the information available on the tubule transport process comes from *in vitro* microperfusions of snake (*Thamnophis* spp.) renal tubules. These studies indicate that transport from peritubular fluid to luminal fluid occurs against a concentration gradient throughout the proximal tubule but not in the distal tubule (375, 378). There is no net reabsorption, but there is a significant passive unidirectional backflux throughout the proximal tubule (Fig. 30) (375).

Net secretion in these snake tubules involves transport into the cells against an electrochemical gradient at the basolateral side and movement from the cells into the lumen down an electrochemical gradient (375). The step at the basolateral membrane appears to involve sodium-independent countertransport for another unknown anion (Fig. 30) (278, 392, 1260, 1510). This step is completely separate from that for other commonly secreted organic anions (e.g. *para*-aminohippurate) (278, 382, 392). So far, there is no convincing evidence from studies on these isolated snake tubules or on brush border membrane vesicles from snake kidneys that the movement of urate from the cells to the tubule lumen is mediated in any way (Fig. 30) (107, 389, 1260). All the data are compatible with simple passive diffusion, but, given the

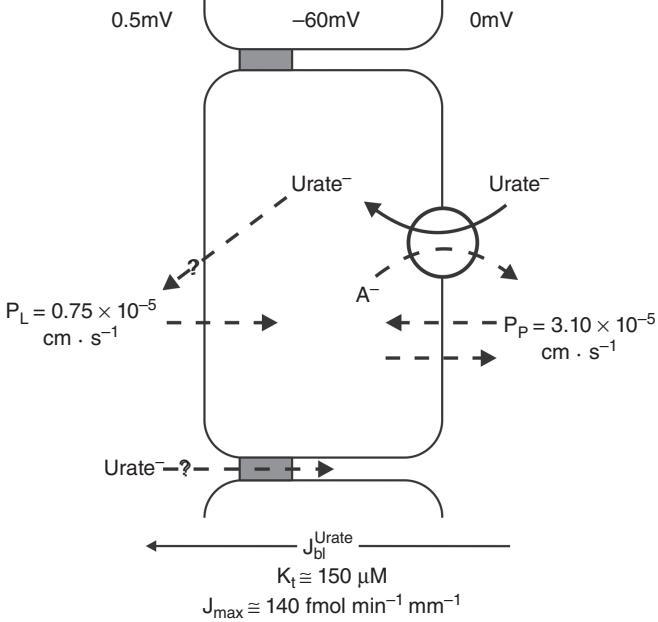

Figure 30 Model for net tubular secretion of urate based on studies with snake (*Thamnophis* spp.) proximal renal tubules. Open circle with solid arrow indicates either primary or secondary active transport. For countertransport, solid arrow indicates movement against electrochemical gradient, and broken arrow indicates movement down electrochemical gradient. Broken arrows with question marks indicate possible passive movements. A^- indicates anion of unspecified nature. Apparent permeabilities of luminal (P_L) and peritubular (P_P) membranes are shown. Apparent K_t and J_{max} for net secretion are shown at bottom of the figure. Reproduced from Dantzler and Bradshaw (392) with permission.

large urate flux across this membrane during net secretion, simple passive diffusion appears highly unlikely (392).

There are a number of other unusual features of transepithelial urate transport in snake renal tubules, which taken together may have adaptive significance (392). First, the apparent passive permeability of the basolateral membrane (3.10×10^{-5} cm/s) is more than four times that of the luminal membrane (0.75×10^{-5} cm/s) so that transport into the cells against an electrochemical gradient at the basolateral membrane is always working against a significant backleak (Fig. 30) (393). Second, function of the basolateral transport step appears to require filtrate (or an artificial perfusate) flowing along the tubule lumen (375). Third, net transepithelial secretion varies directly with the rate of luminal perfusion, reflecting the large transepithelial backdiffusion at low perfusion rates (375, 383). Moreover, the passive transepithelial permeability determined experimentally from this transepithelial backdiffusion (2.4×10^{-5} cm/s) (375, 378) is about four times the value calculated from the independently measured individual membrane permeabilities (0.60×10^{-5} cm/s), indicating that much of this backdiffusion occurs by a paracellular route (378). Finally, the apparent K_t measured for net transepithelial secretion (about 150 μmol/l) (Fig. 30) is significantly below the normal plasma urate concentration (about 400-500 μmol/l), indicating that the transport system is normally saturated (or nearly saturated) (382). This suggests that changes in plasma urate concentration have little effect on net urate secretion. Instead, net secretion (and thus excretion) appears to depend much more on flow rate through the lumen and backdiffusion (382, 392). Given the low solubility of urate, these characteristics of urate transport may be very important in relation to changes in the number of filtering nephrons during dehydration and hydration. The relatively high passive permeability of the basolateral cell membrane, the apparent dependence of transport into the cells across the basolateral membrane on the presence of flow along the lumen, and the apparent large paracellular backleak during low luminal flow rates all may function together to prevent the accumulation of urate in the cells or tubule lumens of nephrons that are not filtering (382, 392). In turn, these transport characteristics may enhance net urate secretion when most (or all) nephrons are filtering and flow along the nephron lumens is high (382, 392).

Bladder, Cloaca and Colon

The water and electrolyte composition of ureteral urine is modified before final excretion by structures distal to the kidneys. The exact combination of the structures involved (bladder, cloaca and colon) depends on the species. No ophidians or crocodilians have urinary bladders, whereas all chelonians have them. Urinary bladders are also found in a number of lizards and in the one extant rhynchocephalian (*Sphenodon punctatum*). However, studies on water and ion reabsorption by reptilian bladders are far from comprehensive and give variable results. Turtle (*Pseudymys scripta*) and tortoise (*Gopherus agassizii* and *Testudo graeca*) bladders show significant osmotic water permeability both *in vivo* and *in vitro* (97, 203, 204, 395). However, they do not respond to antidiuretic hormone, at least *in vitro* (97) (W. H. Dantzler, unpublished obsv.). Nevertheless, the bladder of the desert tortoise (*Gopherus agassizii*) serves as an important organ for storing water during the dry seasons (856, 1285), as well as a sink for potassium, urea and urates (1233). Indeed, masses of precipitated urates appear to be stored in the bladder of these animals and expelled with large volumes of water when free water is plentiful (W. H. Dantzler, unpublished obsv.). The urinary bladder of turtles also shows significant aldosterone-stimulated sodium reabsorption *in vitro*, a feature that enhances water reabsorption and should be important in post-renal regulation of excretion (97, 204, 1029). However, it should be noted that the bladder of the desert tortoise (*Gopherus agassizii*) has a well-developed sphincter, which not only keeps water tightly held within the bladder but also permits ureteral urine to bypass it and move directly into the cloaca (395) (W. H. Dantzler, unpublished obsv.). This sphincter is absent in freshwater turtles (*Pseudemys scripta*) (395) (W. H. Dantzler, unpublished obsv.). These features certainly help determine the degree to which the bladder and cloaca alter the composition of the ureteral urine.

The information about chelonian bladder function, although far from comprehensive, is much more extensive than that available for *Sphenodon* or for those lizards that have them. The bladder of the large skink (*Tiliqua rugosa*) has been reported to be water permeable *in vitro* (94), but there is no significant information on its function *in vivo*. In the Rhynchocephalian (*Sphenodon* punctatum), no net water movement across the wall of the bladder was observed *in vivo* (1627). In some lizards with bladders (e.g., *Hemidactylus fluviatilis*), there is a well-developed bladder sphincter (1651) like that in the desert tortoise and ureteral urine may frequently bypass the bladder (392).

Cloacal and colonic function in modification of ureteral urine and, thus, maintenance of water and electrolyte balance has been studied in some detail only in lizards and, to a lesser extent, in crocodilians (392), although an early study indicated that one species of snake (*Xenodon* sp.) would die within a few days if ureteral urine was not allowed to enter the cloacal-colonic complex (859). A recent comparative morphological and histochemical study of the colon and cloaca of water snakes from marine (*Nerodia clarkia clarkia*) and freshwater (*N. fasciata*) habitats showed no differences between the two species in cell structure or in the distribution of Na^+-K^--ATPase and Na^+-K^+-$2Cl^-$ cotransporter (57). These data suggest that whatever role these factors play in colon or cloacal modification of ureteral urine, they do not contribute to *N. clarkia clarkia* to function in a marine habitat.

A number of investigators have compared ureteral urine with voided urine in lizards and crocodilians and noted that significant amounts of electrolytes and water were apparently reabsorbed from the cloacal-colon complex (174, 176, 1629). For example, Bradshaw (174, 176) found that in the lizard *Ctenophorus* (= *Amphibolurus*) *ornatus*, the relative osmolar clearance (C_{OSM}/C_{IN}) fell from 29% in the ureteral urine to 1% in the voided urine. From a number of such studies on lizards, Bradshaw (173, 176) concluded that the cloacal-colonic complex reabsorbs about 90% of the fluid presented to it during either a water or saline dieresis. However, sodium reabsorption from the fluid presented to the cloacal-colonic complex decreased from about 99% during a water diuresis to about 35% during a saline diuresis, indicating some form of regulation. Bradshaw and Rice (178) reached a similar conclusion in studies on conscious unanesthetized lizards (*Varanus gouldii*), held at their preferred body temperature, in which they perfused the cloacal-colonic complex *in vivo et situ* with artificial ureteral urine at rates appropriate for delivery of ureteral urine during hydration, dehydration and salt loading. During salt loading, compared with hydration, water reabsorption increased significantly from ~23% to ~40% and sodium reabsorption decreased significantly from ~34% to ~22%. The response to dehydration was essentially the same as during salt loading, with an increased rate of reabsorption of water and a decreased rate of reabsorption of sodium. When Bradshaw and Rice (178) calculated the electrolyte concentrations of the reabsorbate with these treatments, they found that it was hypoosmotic with both dehydration and salt loading and hyperosmotic with water loading, suggesting solute-linked water reabsorption as indicated earlier for crocodiles (*Crocodylus acutus*) (1629).

The mechanisms involved in the observed water and electrolyte reabsorption from the cloacal-colonic complex in reptiles are not well understood. A mecholyl-inhibitable, mucosa-negative transepithelial potential has been recorded across cloaca-colon preparations in a number of snakes, lizards, a tortoise and a crocodilian (97, 99, 104, 859, 1620). This observation is compatible with active sodium transport from the lumen side (mucosa) to the blood side (serosa) of these preparations and suggests that transport of water is coupled to sodium transport, as demonstrated by Bindslev and Skadhauge (137) in the large intestine and coprodium of chickens. However, the details of coupling under different reabsorptive conditions have not been examined. Finally, water reabsorption can be facilitated by differences in colloid osmotic pressure across the cloaca of the desert iguana (*Dipsosaurus dorsalis*), and presumably this could occur in other species.

Salt Glands

Many reptile species have specialized extrarenal glands capable of excreting hyperosmotic salt solutions, thereby contributing to the elimination of excess electrolytes (primarily sodium or potassium) while conserving free water. These glands may be external nasal glands, lachrymal glands or premaxillary, sublingual or lingual glands, depending on the species (392). Although salt glands are present in many species, they have not been found in terrestrial snakes, alligators (*Alligator mississippiensis*), geckoes and the one species of pygopodid lizard that has been studied (392, 1595). To date, they have only been described in one species of terrestrial chelonian (*Testudo carbonaria*) (1410).

The primary cation secreted by reptilian salt glands is clearly related to diet and habitat (see table 10.13 in Dantzler and Bradshaw [392] for a detailed review). Regardless of anatomical site or embryological origin, the salt glands of marine species secrete sodium as the primary cation, frequently at extremely high rates that can vary with the salinity of the water to which they are adapted (356), whereas the salt glands of terrestrial tortoises and lizards generally secrete potassium as the primary cation, reflecting their herbivorous diet (392). There are some exceptions. The salt glands of two terrestrial varanid lizards (*Varanus semiremex* and *V. salvator*), which eat crustaceans and other animals in marine mangrove swamps and take in much salt water, secrete primarily sodium (392, 463, 1235). Of perhaps more interest is the observation that the salt glands of the Galapagos terrestrial iguana, which eat a herbivorous diet primarily of *Opuntia* (prickly pear) cactus, secrete substantially more sodium than potassium (461). Also, the salt glands of some terrestrial lizards (e.g., desert iguana *Dipsosaurus dorsalis*), while normally secreting potassium as the primary cation, can be

stimulated to secrete very large amounts of sodium and even rubidium (1677).

Although less is known about the detailed structure, especially ultrastructure, of reptilian salt glands than of avian salt glands (792), the glands are known to consist of a series of secretory tubules that empty into a duct (392). In those salt glands studied, the cells of the tubule epithelium are least specialized at the blind end of the tubule. They become progressively more differentiated into mitochondrion-rich principal cells with extensive lateral membrane infoldings along the length of the tubule away from the blind end (392). In some species (e.g., *Iguana iguana* and *Tiliqua rugosa*), mucoserous cells are interspersed among the principal cells (392). Because of the many mitochondria and the presence of extensive Na^+-K^+-ATPase along the lateral infoldings in the principal cells (490), they are considered to be the cells involved in electrolyte secretion (392). Bumetanide (a blocker of the Na^+-$2Cl^-$-K^+-cotransporter) and ouabain (a blocker of Na^+-K^+-ATPase) both inhibit sodium secretion by isolated salt glands from *Malaclemys terrapin* (1685). These observations suggest that, as in avian salt glands, the secretory process in principal cells in those reptilian salt glands secreting primarily sodium involves these two transporters. Apparently, chloride enters the principal cells at the basolateral membrane via secondary active transport involving the Na^+-$2Cl^-$-K^+-cotransporter and then moves into the lumen through chloride channels with sodium accompanying it via a paracellular pathway. The energy for the process derives from the Na^+-K^+-ATPase (1684), the amount of which can increase with adaptation to increased secretion (354) (see section on Aves for more detail on the secretory process). This transport can be stimulated by cholinergic activation and by activation of the adenylate cyclase pathway by a number of activators, including vasoactive intestinal peptide and possibly other peptides (e.g., B-type natriuretic peptide) (353, 355, 549, 1684, 1685). There are not yet any definitive data on mechanism by which the sodium concentration increases to significantly hyperosmotic levels along the length of the secretory tubules (392).

The mechanism by which secretion occurs in those reptilian salt glands in which potassium is the primary ion secreted is not at all understood. Studies by Shuttleworth et al. (1686) on potassium secretion by nasal salt glands in the desert lizard *Sauromalus obesus* show that stimulation of secretion can lead to a doubling of Na^+-K^+-ATPase activity without any increase in the size or weight of the gland, and that as much as 68% of the potassium in fluid perfusing the gland can be extracted in a single passage. Indeed, Shuttleworth and Hildebrandt (1684) suggest that production of a secretion containing potassium in excess of 1,000 mmol/l with essentially no sodium in this species and others must require a unique mechanism quite different from that for the sodium secreting glands in either reptiles or birds. Whatever the mechanisms involved, salt glands capable of secreting very high concentrations of either sodium or potassium certainly play an important role in regulating the internal environment of many reptile species.

Aves

Birds successfully occupy all habitats on earth, and, like mammals, their water and electrolyte *milieu intérieur* is relatively tightly controlled. To do this, they have evolved a number of physiological mechanisms to assist in osmoregulation and excretion. These mechanisms involve the kidneys, lower gastrointestinal tract and nasal salt glands, which are considered in that order in this section.

Function and Regulation of the Physiological Mechanisms for Osmoregulation

Kidneys

The kidneys play a critical role in regulating the composition of the internal environment of birds, as they do in other tetrapod vertebrates, by controlling the excretion of water, ions and nitrogenous wastes. However, in all birds, substantial modification of the urine occurs distal to the kidneys in the lower gastrointestinal tract. Moreover, in marine species, nasal salt glands contribute to the excretion of ions (primarily sodium) in excess of water. Nevertheless, the kidneys are quantitatively very important in regulating the output of ions and water and, therefore, the composition of the internal environment. This regulation involves adjustments in both filtration at the renal glomerulus and in reabsorption and secretion along the renal tubules.

General form of kidneys and nephrons

The kidneys of birds vary less among species in external gross appearance than those of amphibians, reptiles and even mammals. In all species, the paired kidneys are prominent retroperitoneal organs, which are flattened and recessed into the bony synsacrum (fused lumbar, sacral and caudal vertebrae). They are crossed by the major blood vessels and nerve trunks, binding them tightly into this bony concavity. Each kidney is divided into three divisions—cranial, medial, and caudal—which, depending on the species, may or may not be obvious superficially (853).

The avian kidney consists of cortical and medullary zones, but they are not as clearly demarcated as in mammals. Moreover, it contains two general types of nephrons. The most numerous type (e.g., about 90% in Gambel's quail, *Callipepla gambelii*; about 70% in European starlings, *Sturnus vulgaris*) is short, lacks a loop of Henle and is often referred to as a "reptilian-type" or "loopless" nephron. These are found in the superficial or cortical region of the kidney (Fig. 31). These nephrons, which are simpler than true reptilian nephrons are, consist of a small glomerulus, no neck segment, a proximal tubule with a few simple folds, no intermediate segment and a distal tubule with only one main fold before it empties at right angles into a collecting duct (Fig. 31) (187, 1988). In some of these nephrons, there is a short connecting segment between the distal tubule and the collecting duct

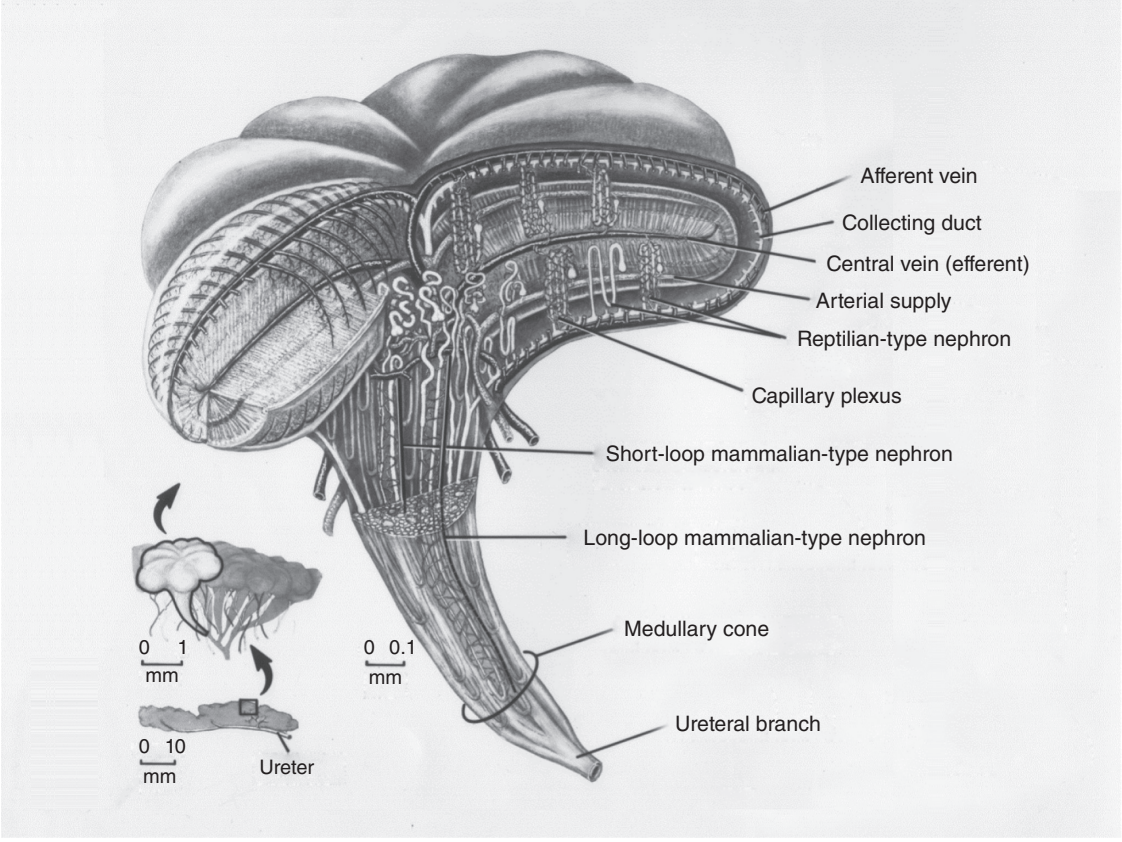

Figure 31 Illustration of detailed internal organization of avian kidney. The whole kidney is shown at the lower left with two successive enlargements of sections of the kidney showing increasing internal detail. The largest section shown gives detail of nephrons forming a single medullary cone. Near the surface of the kidney are the small loopless reptilian-type nephrons arranged in a radiating pattern around a central vein to form a single lobule. In the deeper regions of the cortex are the larger mammalian-type nephrons with highly convoluted proximal tubules, loops of Henle and distal tubules. A gradual transition occurs from loopless reptilian-type nephrons to longest-looped, mammalian-type nephrons. The loops of Henle are in parallel with collecting ducts and vasa recta within a medullary cone, an arrangement that allows countercurrent multiplication (see text). Reproduced from Braun and Dantzler (187) with permission.

(1988). The second, less numerous nephron type (about 10% in Gambel's quail; about 30% in starlings) is located deep in the cortex and extends into the medulla (Fig. 31). These nephrons have loops of Henle, resemble short-looped mammalian nephrons and are referred to as "mammalian-type" or "looped" nephrons. They consist of a glomerulus, which is larger than that of the reptilian-type nephrons, a convoluted proximal tubule, a straight proximal tubule, thin descending limb of Henle's loop, a thick ascending limb of Henle's loop that always begins on the descending side before the bend (prebend region), a distal convoluted tubule and possibly a short terminal connecting tubule before they enter collecting ducts (Fig. 31). It is important to note, however, that the transition from the most superficial reptilian-type to the mammalian-type nephrons is gradual so that there are nephrons of intermediate size and intermediate loop length in the region between the superficial cortical lobules and the deeper medullary region. In all nephrons, the initial portion of the distal tubule in each nephron appears to be tightly apposed to the vascular pole of its own glomerulus, as it is in amphibians, reptiles and mammals. Renin-producing granular cells are found in the glomerular arterioles of all four of these vertebrate classes. However, avian nephrons, in contrast to amphibian and reptilian nephrons and like mammalian nephrons, also appear to have macula densa cells (1250, 1726) and, thus, possibly a complete juxtaglomerular apparatus.

The loops of Henle of the mammalian-type nephrons, vasa recta, which arise from the efferent arterioles of glomeruli of these nephrons, and the collecting ducts, which drain both the reptilian-type and the mammalian-type nephrons from a single radial group of cortical lobules, are arranged in parallel and bound together by a connective tissue sheath into a tapering structure referred to as a medullary cone (Fig. 31). As in the mammalian kidney, the parallel arrangement of loops of Henle, vasa recta and collecting ducts suggests the capability of producing urine hyperosmotic to the plasma (see below). Indeed, the gross structural features of the loops and collecting ducts and their arrangement in the medullary cones are similar to the outer medulla of the developing neonatal rat kidney (1082). However, unlike the mammalian kidney, there is no renal pelvis. The collecting ducts coalesce within this connective tissue sheath to form a single branch of the ureter

(Fig. 31). The number of medullary cones, and thus the number of organized units of the kidney (see Fig. 31 for a single unit) per kidney, is relatively constant for a given species but varies markedly among species (853).

Like amphibian and reptilian kidneys, avian kidneys have both arterial and renal portal blood supplies. The extent to which venous portal blood supplies the kidney is determined by a smooth muscle valve located in the external iliac vein, which is under autonomic nervous control (227). When the valve is closed, portal venous blood contributes to the peritubular capillary blood supply of the reptilian-type nephrons in the cortical region of the kidney (no venous portal blood enters the medullary cones); when the valve is open, venous portal blood bypasses the kidney.

Glomerular function

As in glomerular nephrons of other vertebrates, the initial process in urine formation is the production of an ultrafiltrate of the plasma at the renal glomerulus. This ultrafiltrate supplies water and small solutes to the proximal tubule in the same concentrations as in the plasma. As in other vertebrates, the forces involved in this process are assumed to be the same as those described in detail for reptiles (see above). However, there have been no direct measurements of pressures within the avian kidney. But because birds, like mammals, maintain a high stable arterial blood pressure, the pressures at the glomerulus may be similar to those found in mammals.

However, the capillary network within avian glomeruli is much simpler than that in mammalian glomeruli and the diameter of the avian glomerular capillaries is twice that of mammalian ones (183). Indeed, the network is simpler than that found in reptiles (2056). The glomerular network of the small, loopless, reptilian-type avian nephrons consists of only a couple of loops of a single unbranched capillary (183). The glomerular network of the larger, looped, mammalian-type nephrons consists of a few more loops and may have one or two connecting branches, very different from the highly branched network in mammalian glomeruli (183). Braun (183) suggests that the simple network of capillaries with large diameters is related to the large, nucleated, rigid, nondeformable avian red blood cells.

There is no information on whether this simple network has any direct effect on the factors involved in the filtration process. However, it seems likely that, although the glomerular capillary diameter is larger than in mammals, the much simpler capillary network would actually make the surface area available for filtration smaller than in mammals. It should also be noted that the filtration barrier appears to be less restrictive to large molecules in avian glomeruli than in mammals because large amounts of protein (about 5 mg/ml) appear in the ureteral urine of birds (183). This greater glomerular permeability in birds than in mammals may result from larger slit pores between podocytes and a smaller negative charge on the glycocalyx of the filtration barrier in avian glomeruli than in mammalian glomeruli (259). The apparent physiological significance of protein in the urine is discussed below.

SNGFRs for both reptilian-type and mammalian-type nephrons in birds and superficial and juxtamedullary nephrons in mammals have been measured by the constant infusion sodium ferrocyanide precipitation technique, and the most superficial nephrons in birds and mammals have been measured by the micropuncture technique (Table 17) (184, 187, 196, 382, 1015, 1865). As shown in Table 1, the average SNGFR values for reptilian-type and mammalian-type nephrons are essentially the same in both European starlings and Gambel's quail, and the average value for mammalian-type nephrons is twice that for reptilian-type nephrons. However, the average SNGFR values for the smallest, most superficial reptilian-type nephrons, the only ones accessible to micropuncture, are only about 1/20th of the average values for reptilian-type nephrons as a group (Table 17). Values for these smallest reptilian-type nephrons, determined by the sodium ferrocyanide technique, are similar to those determined by micropuncture (E. J. Braun, personal communication).

The average SNGFR for superficial nephrons in mammals, measured either by the sodium ferrocyanide precipitation technique or the micropuncture technique, is twice that of the mammalian-type nephrons in birds (Table 17). The SNGFR of juxtamedullary nephrons in mammals, determined by the sodium ferrocyanide technique, is more than three times that of the mammalian-type nephrons in birds. Because both the mammals and birds studied have similar mean arterial pressures, it seems likely that the much lower SNGFRs in the birds compared to the mammals reflect the smaller surface area available for filtration in the simple glomerular capillary network in birds. Despite the fact that the SNGFR of bird nephrons is much less than that of mammalian nephrons, the whole-kidney GFR of birds is essentially the same as that of mammals of the same body mass because bird kidneys have many more nephrons than mammalian kidneys (240).

The whole-kidney GFR in birds increases with a water load and decreases with a salt load or dehydration (186, 187, 386, 1561, 1703). Measurements of SNGFRs in Gambel's quail indicate that the decrease in whole-kidney GFR results from a decrease in the number of filtering reptilian-type nephrons with no change in the SNGFR of those filtering (187). At this time, all mammalian-type nephrons continue filtering at the control rates (187). Further studies indicate that this decrease in number of filtering nephrons can be duplicated by physiological doses of the natural avian antidiuretic hormone, arginine vasotocin (AVT), and that the effect involves constriction of afferent arterioles of the nephrons that cease filtering (182, 185). This decrease in the number of filtering reptilian-type nephrons, as in reptiles, can help conserve water at the expense of excreting waste products. It appears to be a reasonable physiological response to dehydration because these nephrons are not arranged to function in concert to contribute directly to the production of hyperosmotic urine. Moreover, as in reptiles, the renal tubules of the reptilian-type nephrons that have ceased filtering can continue to be

Table 17 Examples of Single Nephron Glomerular Filtration Rates (SNGFR) (1264)

Species and Habitat	Nephron Type	SNGFR nl min^{-1}	References
Birds			
Galliformes			
Gambel's quail			
Callipepla gambelii	Mammalian-type	14.6±0.79 (27)[a]	(187)
Arid, terrestrial	Reptilian-type	6.4±0.25 (41)[a]	(187)
	Smallest Reptilian-type	0.37±0.82 (14)[b]	(377)
Passeriformes			
European starling *Sturnus vulgaris*	Mammalian-type	15.6±0.75 (207)[a]	(184)
Moist, arboreal	Reptilian-type	7.0±0.35 (185)[a]	(184)
	Smallest Reptilian-type	0.36±0.040 (17)[b]	(1015)
Mammals			
Rat *Rattus norvegicus*	Superficial	30.1±2.55 (7)[b]	(196)
Terrestrial	Superficial	36.4±3.5 (4)[a]	(1865)
	Juxtamedullary	51.7±6.7 (4)[a]	

Values are means ± SE. Figures in parentheses indicate number determinations. [a]SNGFRs determined by constant infusion sodium ferrocyanide technique. [b]SNGFRs determined by micropuncture technique.

nurtured by the renal portal contribution to the peritubular capillaries. The increase in whole-kidney GFR with a water load reflects an increase in SNGFRs of both mammalian-type and reptilian-type nephrons while all nephrons are filtering (186). It appears very likely that during a normal state of hydration not all the nephrons are filtering (382).

Regulation of glomerular filtration rates by other endocrine systems (e.g., renin-angiotensin system) or by renal nerves, although frequently suggested, has not been clearly demonstrated in birds. Also, although birds appear to have a complete juxtaglomerular apparatus, the presence of a distal tubule-glomerular feedback system has yet to be demonstrated. Nevertheless, avian kidneys exhibit autoregulation of whole-kidney GFR over a renal arterial perfusion pressure of 60 mmHg to 120 mmHg, and the renal portal system appears to play a role in maintaining effective renal plasma flow (1989, 1990).

Tubule function

The renal tubules modify the initial glomerular filtrate by the processes of reabsorption and secretion, thereby determining the final volume, ionic composition and osmolality of the ureteral urine. The following discussion is limited to tubule transport of sodium, potassium, water and the primary excretory end products of nitrogen metabolism.

Inorganic ion transport *Sodium*. Clearance studies on intact animals suggest that the renal tubules of birds, like those of mammals, are capable of reabsorbing 99% of the filtered sodium (382, 1702, 1703). However, as noted above for reptiles, clearance studies in birds do not include any filtered sodium that is contained in urate precipitates (see below) and, therefore, may actually overestimate the fraction of filtered sodium reabsorbed by the renal tubules. In addition, clearance studies only indicate net sodium transport, not whether both secretion and reabsorption of sodium occur. Moreover, further sodium reabsorption may occur in the cloaca (1702).

Estimates derived from *in vivo* micropuncture studies and *in vitro* microperfusion studies suggest that about 60-65% of the filtered sodium can be reabsorbed over the length of the proximal tubule (205, 1015). The micropuncture studies show that sodium and water are reabsorbed isosmotically and that chloride is reabsorbed at the same rate and to the same extent as sodium (1015). No information is available on the details of the transepithelial reabsorptive process for sodium in avian proximal tubules. However, isosmotic fluid reabsorption in isolated perfused avian proximal tubules is inhibited by ouabain in the peritubular bathing medium, indicating that, as in other vertebrates, the reabsorptive process depends on the action of Na^+-K^+-ATPase in the basolateral membrane to transport sodium out of the cells (205).

In vitro microperfusion of isolated thick ascending limbs from large mammalian-type nephrons of Japanese quail

(*Coturnix coturnix*) indicate that these segments reabsorb significant amounts of sodium and chloride without water (1240). As in mammals, these segments have a very low osmotic water permeability (1240) and a lumen-positive transepithelial potential (about +9 mV), which is dependent on the presence of both sodium and chloride in the lumen and which is inhibited by furosemide in the lumen and ouabain in the peritubular bathing medium (1317). These data suggest that reabsorption of sodium and chloride involves entry into the cells via the Na^+-$2Cl^-$-K^+ cotransporter in the luminal membrane and transport of sodium out of the cells via Na^+-K^+-ATPase in the basolateral membrane as in the thick ascending limb of outer medullary nephrons in the mammalian kidney (1317). Therefore, these segments in mammalian-type avian nephrons, like those in mammalian nephrons, appear to be diluting segments and to function in the urine concentrating mechanism (see below). The early segment of the distal tubule of small reptilian-type nephrons, which is in close contact with its parent glomerulus, also has a small lumen-positive potential (1317) and may reabsorb sodium without water in a similar manner to the thick ascending limb of the mammalian-type nephrons, and therefore may function as a diluting segment in reptilian-type nephrons (see below).

Nothing is known about sodium reabsorption in the late distal tubules or collecting ducts in birds. However, the difference between the percent of filtered sodium reabsorbed in the proximal tubule and the percent apparently excreted in the urine suggests that, in addition to significant reabsorption in the diluting segments, there is reabsorption in late distal tubules and collecting ducts.

Almost nothing is known about endocrine control of tubule sodium reabsorption in birds. AVT has no effect on sodium reabsorption in thick ascending limbs of mammalian-type nephrons isolated and perfused *in vitro* (1240). However, in ducks (*Anas platyrhynchos*), adrenalectomy and administration of corticosteroids suggests that aldosterone and corticosterone, both naturally occurring adrenal corticosteroids, may have physiological roles in stimulating tubule sodium reabsorption (791), but no direct studies on tubule transport have been made. There have also been no studies on neural regulation of sodium transport.

Potassium. Clearance studies on domestic fowl indicate that about 10% to 25% of the filtered potassium is usually excreted in ureteral urine, the percent probably influenced by the amount of potassium in the diet (1703), and actual net secretion may occur. However, interpretation of these clearance studies with regard to potassium transport has the same caveats as mentioned above for sodium transport by the renal tubules.

In vivo micropuncture studies on the proximal tubule of the smallest reptilian-type nephrons in starlings indicate that reabsorption of potassium generally occurs over the accessible portion of the tubule but variability in the amount of reabsorption is much greater than for sodium (1015). Indeed, some punctures suggest that secretion may occur (1015). Secretion of potassium along the proximal tubule would be markedly different from what occurs in mammals and probably other vertebrates, where secretion only occurs in distal nephron segments (377). However, although net proximal tubule secretion cannot be ruled out, the small number of points indicating secretion makes definitive interpretation difficult (1015). There are no direct studies of potassium transport along other segments of avian nephrons, although it seems likely that secretion can occur in distal portions of the nephrons (189). No studies have been performed on the mechanism of potassium transport in avian nephrons.

The data on hormonal regulation of potassium transport are few and incomplete. AVT has no effect on potassium excretion in clearance studies (25, 167). Mineralocorticoids like aldosterone might be expected to stimulate potassium secretion as they do in other vertebrates studied, and indeed, administration of aldosterone and corticosterone to ducks does increase fractional excretion of potassium (789). However, the possible tubule sites and mechanism of action of this effect have not been explored.

Water transport *Water reabsorption*. Water reabsorption by the renal tubules plays a critical role in the overall water balance of birds as it does in other vertebrates. Sometimes it occurs at the same rate as solute reabsorption (as isosmotic fluid) and sometimes it lags behind, depending on the need to conserve or excrete water. As in the case of sodium, clearance studies indicate that 99% of the filtered water can be reabsorbed by the renal tubules (377, 1702, 1703). However, in many circumstances a good portion of the filtered water actually is recovered in the lower intestine (see below).

As noted above, micropunctures of the proximal tubules of the superficial reptilian-type nephrons indicate that sodium and water are reabsorbed together in an isosmotic solution (1015). Therefore, as is true for sodium (see above), 60-65% of the filtered water is reabsorbed along the proximal tubule (205, 1015). The reabsorption rate at this time is about 2 nl min^{-1} mm^{-1} in isolated perfused proximal tubules of transitional short-looped mammalian-type nephrons of domestic chickens (205), whereas it is estimated to be about 0.35 nl min^{-1} mm^{-1} in the micropunctured smallest loopless reptilian-type nephrons of starlings (1015). These differences reflect differences in size and filtration rates of these nephron populations (184, 187, 1015).

The isosmotic water reabsorption in avian proximal tubules depends completely on sodium reabsorption, as demonstrated by its inhibition by ouabain in isolated perfused proximal segments (see above) (205). Therefore, it is influenced by anything that influences sodium reabsorption. The details of the process by which water is coupled to sodium reabsorption in avian proximal tubules have not been examined, but the water movement across the epithelium is certainly facilitated by the presence of the water channel aquaporin 1 (AQP1) in both the luminal and basolateral membranes (261).

Table 18 Examples of Range of Urine-to-Plasma (U/P) Osmolality Ratios

Species	Osmolal U/P	Environment and Mode of Existence	References
Chicken	0.1-2.0	Moist, terrestrial	(25)
Gallus gallus domesticus		Gallinaceous	(386)
			(1703)
Gambel's quail	0.5-2.5	Arid, terrestrial	(186, 187)
Callipepla gambelii		Gallinaceous	

Measurements made on ureteral urine.

Dilution and concentration. Reabsorption of water beyond the proximal tubule varies depending on the need to excrete or conserve water. Studies on intact birds indicate that they are all capable of producing dilute urine, the most dilute urine (lowest urine-to-plasma osmolality ratio) generally being found in species with the most access to fresh water (see examples in Table 18) (377). The maximum dilution appears to be as great as that found in any other vertebrates (377). As discussed in the section on reptiles (see above), dilution requires the reabsorption of solute (primarily sodium chloride) without accompanying water in some segment or segments of the nephrons distal to the proximal tubule. As described above, this process apparently can occur in the thick ascending limb of Henle's loop (including the prebend segment) of the looped mammalian-type nephrons and possibly in the early portion of the distal tubule of small loopless reptilian-type nephrons (1240, 1317). Whether any significant dilution can occur beyond these regions is unknown, although it appears very likely that the collecting ducts are capable of performing some additional dilution (1319). In any case, all nephrons (short- and long-looped and loopless) can contribute to the production of urine hypoosmotic to the plasma.

Birds, like mammals and unlike all other non-mammalian vertebrates, are capable of producing urine hyperosmotic to the plasma, but this ability is rather limited (maximum U/P osmolality: about 2.0-2.5) (see, for example, Table 18) (189, 377). This urine-concentrating ability is related to the presence of nephrons with loops of Henle arranged parallel to collecting ducts and looped capillaries in the kidneys of birds and mammals (377) and the resulting production of an increasing osmotic gradient along the length of the medullary cones in bird kidneys (491, 1704) and the medulla in mammalian kidneys (377). In contrast to mammalian kidneys, however, in which this osmotic gradient involves both sodium chloride and urea, in avian kidneys the gradient consists almost entirely of sodium chloride and, to a very slight extent, potassium chloride (1704). Nevertheless, as in mammalian kidneys, the generation of this gradient appears to involve countercurrent multiplication (377).

On the basis primarily of studies of tubule transport characteristics in Japanese quail, Nishimura et al. (1318) proposed that countercurrent multiplication occurs by single-solute recycling. They proposed that sodium chloride transported out of the thick ascending limb without accompanying water (see above) is the single effect driving the process, and that this sodium chloride is recycled by entering the thin descending limb, which has very high sodium and chloride permeabilities and very low water permeability (1318). Nishimura et al. (1318) further suggested that this process combined with a cascade of loops of increasing lengths could establish the sodium chloride-based osmotic gradient in the medullary cones. In the presence of AVT this osmotic gradient would then cause water to move out of the parallel collecting ducts, thereby concentrating the urine to the level of the osmotic gradient (1318). As in the outer medulla of the mammalian kidney, the thick ascending limbs would carry fluid that is hypoosmotic to the plasma from the medullary cones into the cortical region. An aquaporin 2 (AQP2) water channel, which is a homologue of mammalian antidiuretic hormone-responsive AQP2, is present in the apical membrane and subapical cytoplasmic region of quail collecting duct cells, and it appears to increase in the apical membrane in the presence of AVT (2053). However, the osmotic water permeability of quail collecting ducts is low and only increases a modest amount in the presence of AVT (1319). Nevertheless, a mathematical simulation of this proposed model (using the anatomical and transport characteristics of quail nephrons) generated a maximum U/P osmolality ratio of about 2.26, a value consistent with what is actually observed in birds (e.g., Table 18) (1021). This mathematical model also indicated that active sodium chloride transport out of the prebend region of the thick ascending limb is of great significance in producing the osmotic gradient. Moreover, further mathematical modeling (1020) indicated that the effectiveness of the avian concentrating mechanism depends in part on the structural arrangement of tubules and vessels within the medullary cones (a ring of collecting ducts surrounding a core of thin limbs and capillaries, with thick limbs distributed inside and at the periphery of that core) described by Nishimura et al. (1318). The limited concentrating ability of the avian kidney is significant with regard to urate excretion (see below) and water reabsorption in the lower intestinal tract (see below) and does not reflect the overall ability of birds to conserve water.

Table 19 Examples of Approximate Percent Total Urinary Nitrogen as Urates, Urea, and Ammonia

	Percent urinary nitrogen as:			
	Urates	Urea	Ammonia	References
Chicken Gallus gallus domesticus (O)	55-72	2-11	11-21	(1212)
Duck Anas platyrhynchos (O)	54	1	29	(1762)
Anna's Hummingbird Calypte anna (N)	35-65	8-13	27-53	(1480)
Turkey Vulture Cathartes aura (C)	76-87	4	9-16	(1213)

O, omnivore; N, nectivores; C, carnivore.

Excretory End Products of Nitrogen Metabolism: Ammonia, Urea and Uric Acid

Birds are generally considered to be uricotelic—that is, urate is the primary form in which excess nitrogen is excreted (Table 19). However, a significant fraction (average about 20%) of urinary nitrogen is in the form of ammonia in the urine of most birds, and at least one nectivorous species (Anna's humming bird, *Calypte anna*) can excrete over 50% as ammonium salts when its intake of nectar is high at low temperatures (Table 19) (1480). Thus, this species and perhaps some other nectivores appear to be able to change from being uricotelic to ammoniotelic (i.e., they are facultative ammoniouricoteles) (Table 19). In all avian species, urea accounts for only a very small percentage of the nitrogen excreted in the urine (Table 19).

Ammonia. Because ammonia is highly toxic, very little is found in the blood and, therefore, almost none is filtered. Instead, as in other vertebrates, it is produced and secreted by the renal tubules themselves. Although ammonia appears to play a significant role in nitrogen excretion in birds (and the major role in a few species), its production and excretion are also likely to be very important in maintaining acid-base balance by removing acid and generating an equivalent amount of bicarbonate. Indeed, the production and excretion of ammonia have been shown to increase with an acid load in chickens (*Gallus gallus domesticus*) (346, 1093).

Although the exact tubule site of ammonia production in birds is unknown, the appropriate enzymes for its production are found in homogenates of the whole chicken kidney and in tubules separated from superficial regions of the kidney, apparently from reptilian-type nephrons (346, 347). The activities of glutaminase I, glutamate dehydrogenase, and alanine aminotransferase increase in the kidneys of acidotic chickens (346). In addition, Craan et al. (346) found that the production of ammonia is increased in superficial tubules from acidotic chickens compared with those from control chickens when they are incubated *in vitro* with either alanine or glutamine. However, because only the uptake of glutamine, not alanine, is enhanced in tubules isolated from acidotic animals, Craan et al. (347) suggested that glutamine is the preferred substrate for enhanced ammonia production.

Neither the tubule site nor the mechanism of ammonia secretion is known. However, as discussed above with regard to reptiles, secretion could involve both non-ionic diffusion of free ammonia (NH_3) with trapping as ammonium (NH_4^+) in the lumen and carrier-mediated transport of ammonium ions (NH_4^+), depending on the pH of the luminal fluid and the quantity of ammonia to be secreted. Because micropuncture studies on starlings have indicated that the pH of the luminal fluid is about 7.6 in the proximal tubules and about 6.4 in early cortical collecting ducts (1013), it seems likely that carrier-mediated transport of ammonium ions would be most important in the proximal tubule where much of the ammonium is likely to be produced, whereas non-ionic diffusion could play a significant role in the collecting ducts. In any case, ammonia is actually excreted in the urine as ammonium salts, probably either ammonium chloride or, in these uricotelic animals, ammonium urate. It should be added that although ureteral urine may enter the colon before it is excreted (see below), recent studies have shown that the colonic epithelium has an extremely low permeability for NH_3 and is also capable of secreting NH_4^+ into the colon lumen mediated by a vacuolar type H^+-ATPase (794). Thus high ammonia concentrations can be maintained.

Urate. Most uric acid is produced in the liver, exists as urate salts in the plasma, is filtered by the glomerulus and is secreted by the renal tubules. Although urates appear to be freely filtered by the avian glomeruli, there is some evidence that a small amount is bound to plasma proteins (387). In addition, variable amounts of urate can be synthesized by the renal tubules. In fasted chickens, such urate apparently contributes about 3% of that excreted, in normally fed chickens about

20% and in chickens systemically infused with hypoxanthine about 50% (284).

Although urate is filtered, net secretion by the proximal tubules contributes most of the urate in the avian urine (final excretion of about five times the amount filtered) (1016). Despite some urate production in the renal tubule cells, most of this secreted urate involves transepithelial transport from the peritubular blood to the tubule lumen. *In vivo* micropuncture studies on superficial loopless reptilian-type nephrons in starlings and *in vitro* microperfusion studies on transitional looped nephrons in chickens clearly show that net secretion occurs along the proximal tubules (205, 1016). Studies with isolated, perfused and non-perfused chicken proximal tubules and primary cell cultures indicate that the net secretion involves uptake into the cells against an electrochemical gradient and movement from the cells into the lumen down an electrochemical gradient (205, 455). Inhibitory studies suggest that much, but not all, of the urate transport into the cells at the basolateral membrane involves the same pathway as para-aminohippurate (PAH) and other organic anions and involves countertransport for α-ketoglutarate (αKG) (Fig. 32). However, at least a portion appears to involve some other pathway with probable countertransport for another unknown anion (Fig. 32). In mammals, the basolateral exchange of organic anions (e.g., PAH) for αKG involves the organic anion transporters (OATs). Since mRNA for OAT-like organic anion transporters has been found in chicken proximal tubule epithelium (455), it seems likely that OAT orthologs are involved in the basolateral transport of urate and other organic anions (e.g., PAH) in avian proximal tubules (Fig. 32). There is some unilateral backflux of urate from lumen to peritubular fluid, and because no urate accumulates in the tubule cells during this process, it seems likely that this backflux moves between the cells (Fig. 32) (205).

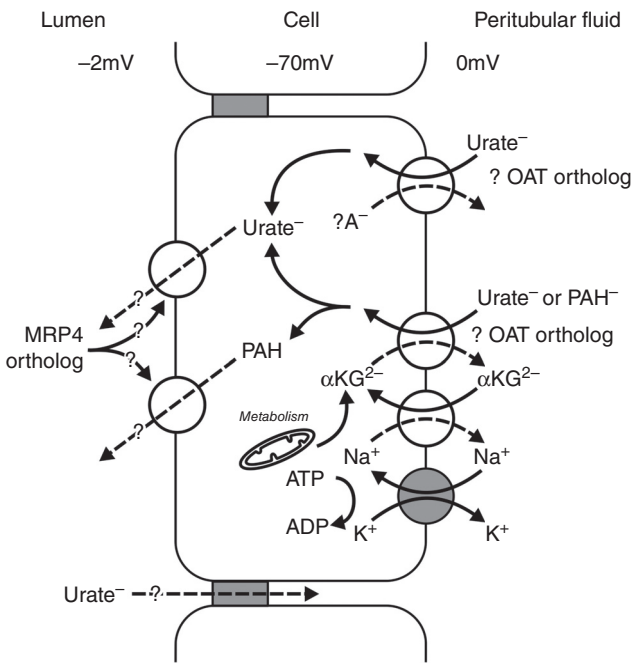

Figure 32 Model for net tubular secretion of urate and PAH in avian proximal tubules, based on studies in chickens. Filled circle with solid arrow indicates primary active transport. Open circle with solid arrow indicates secondary active transport. Open circle with broken arrow indicates carrier-mediated passive transport. Therefore, for countertransport, solid arrow indicates movement against electrochemical gradient; broken arrow indicates movement down electrochemical gradient. A^- indicates anion of unspecified nature. Question marks indicate that these processes have not been clearly demonstrated.

Although the transport step for both urate and PAH from the tubule cells to the lumen in birds is down an electrochemical gradient, little is known about it. However, mRNA for a multidrug resistance peptide 4 (MRP4)-like transporter is found in chicken tubule epithelium, and inhibitor studies suggest that it may be involved in the process (Fig. 32) (455). If this is the case, of course, then this luminal flux would not be completely passive because this is an ATP-dependent transporter.

Although the concentration of urate in avian plasma is well within the solubility range of urate salts (see section on Reptiles for solubilities), the concentrations developed in the lumens of the renal tubules exceed even that for potassium urate and are well above those for sodium urate or ammonium urate (1016). Although there is visual evidence for an apparent urate precipitate in the collecting ducts of starlings (1016), any precipitation in the proximal and distal tubules of avian nephrons is prevented by binding to serum albumen, which, as noted above, is filtered to a significant extent in birds (183, 259, 1758). Urate and its associated albumen form small spheres (0.5-0.14 mm in diameter) (190) beginning in the early part of the proximal tubule (245, 260). Because of the large amounts of protein, these spheres are held in a colloidal suspension in the tubule lumen (190) so that precipitation only begins to occur in the more concentrated urine of the collecting ducts (1016). However, the limited urine concentrating ability of the avian kidney (see above) probably prevents much greater precipitation before the urine enters the lower gastrointestinal tract. As noted above in the discussion on reptiles, the inorganic cations of the urate salts trapped in colloid suspensions or in these precipitates would not contribute to the osmolality of the aqueous phase of the ureteral urine, and therefore the limited concentrating ability of the kidney does not accurately reflect its ability to excrete inorganic cations in the ureteral urine.

The large amount of protein associated with urate is actually reclaimed in the lower gastrointestinal tract (see below). In this region, bacteria break down the protein, allowing the resulting peptides and amino acids to be reabsorbed by the colonic epithelium (245, 260), and no protein appears in the urine that is finally excreted (841). Finally, about 60% of the uric acid entering the colon is also broken down, permitting reabsorption and reutilization of nitrogen, if necessary, as may be the case in nectivorous and frugivorous species (30, 183, 883).

Lower Gastrointestinal Tract

Substantial modification of the ureteral urine can occur in the lower gastrointestinal tract (coprodeum and colon) and this has been extensively studied, particularly by Skadhauge and his colleagues (e.g., see the following reviews [620, 1017, 1018]). Most of the studies were undertaken on domestic fowl (*Gallus gallus domesticus*), but similar results have been observed in other species. Only a few aspects are mentioned in this review. The ureteral urine first enters the urodeum and then moves retrograde driven by peristaltic movements first into the coprodeum and then into the colon (and cecae in those species in which they are found) (183). The extent of this retrograde peristalsis from the coprodeum into the colon appears to be regulated in part by the sodium concentration and osmolality of the ureteral urine, being marked at low osmolalities and low sodium concentrations but tending to cease when the osmolality is more than 100 mOsm above the osmolality of the plasma (211). This is significant with regard to solute-linked water reabsorption in the colon (see below).

Both the coprodium and colon are capable of reabsorbing sodium chloride, but this process is influenced by sodium chloride intake and aldosterone. Moreover, there are distinct differences in the transport processes between the coprodeum and the colon. Most of the studies on the sodium transport process have been performed *in vitro* where, under voltage clamp conditions, the short-circuit currents (I_{SC}) are virtually identical to active sodium reabsorption, as determined by isotopic flux measurements (292, 1065, 1066), but comparable data are observed *in vivo*.

Coprodeum. When birds are maintained on a high sodium chloride diet or a medium sodium diet (such as that found in commercial feed), sodium reabsorption and I_{SC} in this region are essentially zero. However, when the animals are adapted to a low sodium chloride diet, sodium is reabsorbed at a very high rate (net sodium flux: 14 $\mu Eq\ cm^{-2} \cdot hr^{-1}$; I_{SC}: 380 $\mu A \cdot cm^{-2}$) (51, 292, 304). During this high-sodium transport under open-circuit conditions *in vitro* and *in vivo*, a lumen-negative transepithelial potential difference of 40-60 mV develops and drives passive chloride reabsorption to accompany the sodium. This dramatic change from almost no sodium reabsorption during adaptation to a high-sodium diet to a very high rate of sodium reabsorption during adaptation to a low-sodium diet results from activation of amiloride-sensitive sodium channels (ENaCs) in the luminal membrane (134, 296, 305). The energy-requiring sodium transport step at the basolateral membrane involves Na^+-K^+-ATPase, which does not change with changes in sodium chloride in the diet (1190). Stimulation of sodium transport similar to that produced during a low-sodium diet can be produced by aldosterone, the plasma level of which is maximal with adaptation to a low-sodium diet and nearly undetectable with adaptation to a high-sodium diet (51, 303, 304, 1093, 1700). Finally, there is no absorption of glucose or amino acids and no solute-linked water flow in the coprodeum (135, 1539).

Colon. Sodium reabsorption in the colon, as in the coprodeum, changes with adaptation to different sodium diets. However, the processes involved are substantially more complex. During adaptation to a low-sodium diet, sodium reabsorption, as in the coprodeum, involves an electrogenic, ENaC-mediated process of similar magnitude. Also, as in the coprodeum, there is no stimulation of sodium transport by amino acids or hexoses. However, the epithelium of the colon is leakier than that of the coprodeum (resistance of about 80-100 $ohm \cdot cm^2$ versus 300-500 $ohm \cdot cm^2$) and, therefore, has a lower transepithelial PD (15-20 mV versus 40-60 mV). Along with this, there is substantial solute-linked water reabsorption that can occur against an osmotic pressure gradient of 100-180 mosmol/kg (lumen > blood) (137, 618, 619, 1539, 1705). Therefore, as long as ureteral urine does not have an osmolality more than about 100-180 mosmol/kg greater than the plasma, it will move retrograde (see above) into the colon, and the solute-linked water reabsorption will offset movement of water into the hyperosmotic lumen. More concentrated urine, which at most is only about 300 mosmol/kg above the plasma osmolality, will remain in the coprodium before excretion.

With adaptation to a high-sodium diet, ENaC channel activity in the luminal membrane almost completely disappears, as it does in the coprodeum. However, in the colon, sodium reabsorption continues with the entry step across the luminal membrane involving a number of other processes. The most important of these are apparently sodium-coupled glucose and amino acid cotransport (51, 303, 1065, 1066, 1538). Sodium-glucose cotransport is attributed to one of the sodium glucose luminal transporters (SGLTs) (136, 1014). Thus, most of this continued sodium transport is dependent on the presence of glucose and amino acids in the lumen. At the same time, these processes help reclaim glucose and amino acids still present in the ureteral urine and in the chyme moving down the gastrointestinal tract.

In addition to entry via these cotransporters, sodium can cross the luminal membrane via sodium-hydrogen countertransport, apparently involving the sodium-hydrogen exchanger 2 (NHE2) isoform (403, 444). Overall, the colon has a much higher total transport capacity than the coprodeum. This suggests that the colon does much of the work of sodium and water reabsorption from ureteral urine and chyme, whereas the coprodeum functions to fine tune the sodium excretion (1821).

Caeca. Not all bird species have caeca. However, *in vivo* and *in vitro* studies of transport in the main body of caeca of domestic fowl have revealed substantial amiloride-sensitive reabsorption of sodium (670, 671, 1538, 1823). Moreover, high rates of solute-coupled water reabsorption accompany the sodium reabsorption, indicating that the caeca may play an important role in overall solute and water balance (1538, 1822, 1823). As in the coprodeum and colon, the reabsorption of sodium is enhanced by adaptation to a low sodium diet and by aldosterone (671, 1823). Dehydration can also enhance net sodium reabsorption and its accompanying water flux (1538,

1823). Taken together, these data indicate that the caeca, along with the colon, may play an important role in overall solute and water balance in those species that have them.

Salt Glands

As noted above, the limited urine-concentrating ability of birds is closely related to the need to excrete urate, itself a water-conserving process, and the solute coupled water absorption in the lower intestine. However, in marine birds and birds that can adapt to a marine habitat, the need to excrete sodium chloride and generate free water far exceeds the capabilities of their kidneys. This requirement is met by secretion of a highly concentrated sodium chloride solution by paired supraorbital nasal salt glands, as first described by Knut Schmidt-Nielsen and his colleagues (1631). All birds examined have at least rudimentary salt glands (1159), but their development and functional activity depend on the amount of sodium chloride consumed by the animals (1684).

The gross and fine structure of these glands, as well as their blood supply and innervation, have been well described and reviewed by a number of investigators (232, 602, 803) and will not be discussed in detail here. However, as noted with regard to reptiles (see above), the glands consist of secretory tubules. Groups of these tubules form lobules in which all of the tubules empty into a common central ductule. A number of these ductules empty into primary ducts, which in turn empty into two main ducts, one for each gland. These ducts carry the secreted fluid to the nasal cavity (232) where it is removed by passive dripping or shaking of the head.

As also noted in the section on reptiles (see above) the epithelium of each of the secretory tubules has poorly differentiated, apparently non-secretory cells near the blind end. The cells then become progressively more differentiated along the length of the tubules, developing extensive infoldings of the basolateral membrane, along which Na^+-K^+-ATPase is localized (151, 499, 797), and an increasing density of mitochondria, as expected of ion-transporting epithelium. A hyperosmotic sodium chloride solution, with only minor contributions from other ions, is secreted by these cells in all birds studied when they are given an osmotic stimulus. The process by which the initial secretion is considered to occur involves secondary active chloride transport and is illustrated in Fig. 33. Chloride enters the secretory cells across the basolateral membrane via the Na^+-$2Cl^-$-K^+ cotransporter (500, 1843) and exits across the luminal membrane via chloride channels; potassium is returned to the basolateral fluid via basolateral potassium channels (1175, 1541). Sodium accompanies the chloride via a paracellular pathway (Fig. 33). The active, energy-requiring step, which drives the whole process, involves the basolateral Na^+-K^+-ATPase, which maintains a low intracellular sodium concentration. As in the case of the reptilian salt glands (see above), the process by which the final sodium chloride concentration in the secreted fluid is produced is unclear. Shuttleworth and Hildebrandt (1684) suggest

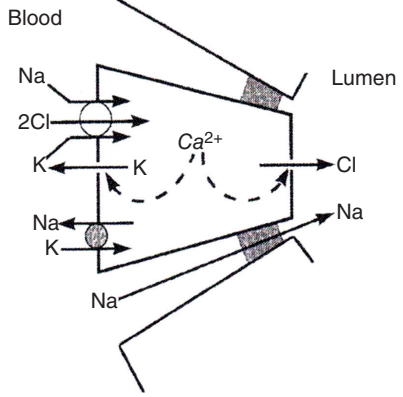

Figure 33 Diagram illustrating the proposed mechanism of secretion in the avian salt gland. Increases in cytosolic Ca^{2+} activate secretion by increasing the opening of basolateral Ca^{2+}-activated K^+ channels and apical Ca^{2+}-activated Cl^- channels. Reproduced from Shuttleworth and Hildebrandt (1684) with permission.

that the most likely mechanism involves hyperosmotic secretion by the cells with no further modification along the ducts. In any case, the concentration of sodium chloride in this solution can reach as much as 10 times that in the plasma, in some species (1411). Moreover, Kaul et al. (904) reported that the glands in saline-adapted ducks are capable of removing more than 20% of the sodium in the blood passing through them. This sodium chloride secretory process in the salt glands of reptiles and birds is essentially the same as that in the rectal glands of elasmobranchs and the gills of marine teleosts (see section on Agnatha and Pisces and Fig. 18).

Regulation of the avian salt glands involves (1) progressive adaptation upon exposure to a salt load and (2) rapid activation of the secretory process in the already salt-adapted gland. Control certainly appears to involve central osmoreceptors as originally suggested by Schmidt-Nielsen (1631), but extra-cerebral osmoreceptors may play some role (697). In any case, the output from these receptors is conveyed to the salt glands via cholinergic fibers in the secretory nerve that stimulates each gland (571, 697). Parasympathetic fibers in this nerve have endings that carry both vesicles filled with acetylcholine (796) and vesicles filled with vasoactive intestinal peptide (601, 1104), which are released upon electrical stimulation. However, as reviewed by Shuttleworth and Hildebrandt (1684), regulation of both of the responses noted above involves neuronal release of acetylcholine, which interacts with muscarinic receptors on the secretory cells. This leads, in turn, via the phosphatidylinositol system, to an increase in the free calcium concentration in the cytoplasm. The way in which this one signaling pathway leads to the two separate responses has been studied by Shuttleworth and his colleagues and is reviewed in Shuttleworth and Hildebrandt (1684). This review should be consulted for further details. In any case, the nasal salt glands are sufficiently effective to permit some species of birds to thrive in areas where little freshwater is available.

Nitrogen Excretion

Origin of Nitrogeneous Compounds

Ammonia

Ammonia (i.e., the total of NH_3 and NH_4^+, T_{Amm}) is present in terrestrial and aquatic environments. It is generated by atmospheric fixation due the breakdown of N_2 by lightning, by decomposition of nitrogenous organic material and by energetically costly nitrogen fixation of microorganisms utilizing the oxygen-sensitive nitrogenase enzyme system (805, 1488). Large amounts of nitrogenous material are produced by anthropogenic activities and a significant proportion of this is eventually converted into ammonia. Anthropogenic ammonia derives primarily from the production and use of fertilizers, biomass burning and from intensive animal husbandry (15, 839).

In addition, animals produce and excrete nitrogenous molecules. The vast majority of these nitrogenous molecules are the three main waste products, namely ammonia, urea and uric acid. Synthesis and pathways of these metabolic endproducts in animals have been extensively described by J. W. Campbell (248) and will therefore be covered only to a minor extent in this article. Most of the nitrogen of the major nitrogenous waste products derives from the degradation of amino acids, in order to utilize the remaining carbohydrate skeleton for energy production or storage as glycogen or triglycerides (968). The main pathway for amino acid catabolism requires an initial transfer of the α-amino group of a given amino acid to α-ketoglutarate by an aminotransferase (also known as transaminase) to form glutamic acid (glutamate) and the corresponding α-keto acid. Glutamate is then transported into the mitochondria, where the amino acid is oxidatively deaminated by glutamate dehydrogenase (193).

Serine and threonine can be deaminated directly by serine-dehydratase and threonine-dehydratase, respectively. Serine-dehydratase is present in mammalian hepatocytes, here almost exclusively in the cytoslic compartment (1787). Serine-dehydratase was also found in invertebrates, for example, in the gills, the digestive diverticula and mantle and foot muscle of the brackish-water bivalve *Corbicula japonica* (716). However, due to its low activity in invertebrates other than molluscs, the role of serine-hydratase in amino acid catabolism and ammonia generation in invertebrates is unclear.

Proline is often used directly as an energy source in invertebrates, for example, in tissues with high oxidative rates such as insect flight muscles (63, 1593) or in the mantle tissues of cephalopods (1782). After the initial conversion of proline to Δ-pyrroline-5-carboxylate, the product is then further converted into glutamate (777).

A second pathway of amino acid metabolism is the so-called purine nucleotide cycle. This system plays supposedly only a minor role in some specific mammalian tissues (1102, 1103). In invertebrates, however, this system might be of more importance. In this mechanism, transaminase reactions are coupled with the purine nucleotide cycle, which is involved in the synthesis of nucleic acids. Here inosine monophosphate (IMP) and aspartate, which is synthesized from oxalacetate and the α-amino group derived from the deamination of glutamate, form adenylosuccinate, a precursor of adenosine monophosphate (AMP). After deamination of AMP by AMP deaminase, ammonia is released and IMP enters the cycle again. As reviewed by Campbell, bivalve and gastropod molluscs have high aspartate animotransferase and AMP deaminase activities but low glutamate dehydrogenase activity (248). Also, adenylosuccinate synthase and adenylosuccinate lyase were found in the hepatopancreas of the gastropod *Helix aspersa* (249), indicating the putative participation of the purine nucleotide cycle in ammonia generation in molluscs.

As in other animals, the vast majority of the excreted ammonia in crustaceans originates from the catabolism of proteins and amino acids. Some additional ammonia is produced in reactions involving purine and pyrimidine bases (307, 1950). Ammonia derived via this uricolytic pathway is considered to contribute only a small portion of the total excreted compared to the predominant production from amino acids (705, 1634). Ammonia derives for the most part from deamination of glutamine, glutamate, serine and asparagine by the specific enzymes glutaminase, glutamate dehydrogenase, serine dehydrogenase and asparaginase, respectively (643, 926, 969). The purification and characterization of AMP deaminase from crustaceans (1752) raised the possibility that ammonia could also be produced by deamination of adenylate via the purine-nucleotide-cycle. Further, confirmed by analyzing expressed sequence tags (ESTs), mRNA coding for adenylosuccinate synthase is indeed expressed in tissues of lobster *Homarus americanus* (Genbank accession # EH116452) and the green shore crab *Carinus maenas* (Genbank accession # DN550904). The role of this particular pathway, however, is not clear to date. The amount of metabolic ammonia produced in crab gill epithelium itself is significant. In marine stenohaline *Cancer pagurus* crabs, the excretion due to branchial metabolic ammonia was 12.3 µmol gFW^{-1} h^{-1}. About 50% of these rates were found in gills of the osmoregulating crabs *Carcinus maenas* and *Eriocheir sinensis* (1945). For comparison, the overall excretion rates of total ammonia in *Cancer pagurus*, *Carcinus maenas* and *Eriocheir sinensis* were only ca. 340, 140 and 120 nmol gFW^{-1} h^{-1}, respectively (1945).

Urea

Urea is the primary waste product of adult amphibians, turtles, mammals and some invertebrates. Here toxic ammonia, generated in the mitochondria, is converted into urea for excretion. Besides being a less toxic nitrogenous waste product compared to ammonia, urea is also synthesized for osmoregulatory purposes. While in mammals urea is used in the kidneys to produce a hypotonic urine (385), it serves in other vertebrates as an osmolyte to balance osmotic

gradients between the body fluids and the environment (926). The fully aquatic and ammonotelic African claw frog *Xenopus laevis* becomes ureotelic when acclimated to 400 mosmol·kg^{-1} solutions of NaCl (2031). In marine elasmobranchs, urea is an important osmolyte with blood concentrations of 300 to 400 mmol/l. Urea is synthesized in the ornithine-urea-cycle (OUC), which has been reviewed extensively by Meijer and others (1217). A key enzyme in this process is the carbamyl phosphate synthetase (CPS). In animal systems, three CPSs exist: CPS-I, CPS-II and CPS-III. In ureotelic mammals and amphibians, CPS-I utilizes ammonia as a substrate and N-acetyl-l-glutamate for activation. CPS-I functions in hepatic ammonia detoxification (1498). CPS-II does not require N-acetyl-l-glutamate and utilizes glutamine as a substrate (639). CPS-III is involved in pyrimidine synthesis. Similar to CPS-II, the mitochondrial enzyme CPS-III utilizes glutamine as a substrate, but in contrast to CPS-II, it requires N-acetylglutamate. CPS-III is involved in urea synthesis in invertebrates and elasmobranchs, were urea is used as osmotic ballast (32) (258, 1852, 1853). For a more detailed review on the ornithine-urea-cycle and its regulation, see also Campbell (248, 1986). In most invertebrates and teleost fish, urea is synthesized by uricolysis or argininolysis (621, 2040). In general, teleost fish are ammonotelic, and enzymes necessary for a functional urea cycle are suppressed (812). However, some teleost species synthesize fair amounts of urea and can become ureotelic. This can occur in response to environmental conditions that limit ammonia excretion, for example, in the tilapia *Oreochromis alcalicus grahami* (1509) living in highly alkaline lakes (pH ≈ 9.6-10) or the air-breathing Asian stinging catfish *Heteropneustes fossilis* (1594). For the Gulf toadfish *Opsanus beta* it has been speculated that urea production and excretion is used as a protective mechanism to cloak the scent of ammonia that might lure predators (73, 1202). All these species express the full complement of OUC enzymes. In contrast, insects lack one or more genes-encoding enzymes required for a fully functional OUC (2069). Scaraffia and others discovered that in addition to urea production by arginase-catalyzed hydrolysis of dietary arginine, the yellow fewer mosquito *Aedes aegypti* synthesizes urea in an amphibian-like uricotylic pathway (1617).

Uric acid

In arachnids, reptiles, birds and crocodilians, excessive ammonia from amino acid catabolism is mostly converted into uric acid or other purines for excretion. Uric acid is relatively non-toxic and very little water is required for its excretion. Using uric acid as the primary nitrogenous waste product is therefore a major water conservation mechanisms. However, its synthesis is energetically very expensive. Uric acid and other purines are also formed for excretion by land-living snails and arthropods, including arachnids, insects and the terrestrial robber crab *Birgus latro* (645). To date it is not clear whether uric acid synthesis in invertebrates is analogous to the pathways found in the liver of higher vertebrates. Pathways for uric acid synthesis and its regulation have been reviewed in detail by Campbell (248).

Toxicity of Ammonia

Ammonia is highly toxic in animals and at higher levels also in plants (198). With a pK of 9.2 to 9.8, which depends on the temperature and salinity of the media (243), ammonia is a weak base. In body fluids with a physiological pH ranging roughly from 7.2 to 7.8, approximately 95% to 99% of total ammonia occurs in the hydrated form NH_4^+. Both forms of ammonia, NH_3 and NH_4^+, have toxic effects by potentially disturbing the cytosolic and/or intraorganelle pH. For instance, NH_3 sequestered by acidic organelles due to ammonia trapping (see below) can impair proper functioning of Golgi vesicles and lysosomal proteases by shifting the intraorganelle pH away from its optimum necessary for normal operation. Ammonia formed from glutamate deamination is generated within the mitochondria, where its toxicity results from disruption of the proton gradient across the inner mitochondrial membrane. Due to its relative alkalinity (ΔpH mitochondria vs. cytoplasm ≈ 0.8), an outwardly directed P_{NH3} is given. Along this gradient NH_3 exits the mitochondrial matrix and binds to protons in the intermembrane space, thereby abolishing the pH gradient, which drives oxidative phosphorylation. Ammonia thereby acts as an H^+-gradient-uncoupler (1338).

Hydrated NH_4^+ and K^+ ions have the same ionic radius of 1.45 Å (950, 1957) and due to their K^+-like behavior, ammonium ions affect the membrane potential, for example, in the giant axon of *Loligo pealei* (139) and in mammalian neurons (337). Ammonia also exhibits an inhibitory effect on epithelial Na^+ channels (ENaC) when expressed in *Xenopus* oocytes (1293). The mode of inhibition is not fully understood, however.

In mammals, elevated ammonia causes major damage in the central nervous system, including changes in blood-brain barrier morphology (1012). In addition, elevated ammonia levels in mammals have been related to Alzheimer disease (Alzheimer Type II astrocytosis) due to toxic accumulation of glutamine in astrocytes, which leads to cell swelling and cell death (236). Interestingly, in the ammonia-tolerant Gulf toadfish *Opsanus beta*, the hydration status of the brain does not change with increased brain glutamine levels in ammonia-stressed animals (1906, 1929).

In microglia and astroglioma cell lines, ammonia affects major functional activities such as phagocytosis and endocytosis. In addition, ammonia modifies the release of cytokines and increases the activity of lysosomal hydrolases (52, 53). Ammonium ions inhibit important enzymes involved in metabolism, such as isocitrate dehydrogenase and α-ketoglutarate dehydrogenase (337).

Marcaida and coworkers found evidence that ammonia toxicity is mediated by excessive activation of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors in the brain.

As a consequence, cerebral ATP depletes while intracellular Ca^{2+} increases, with subsequent increases in extracellular K^+ and finally cell death (1154). In addition to this, neurotoxicity is mediated by a direct inhibitory effect of ammonia on the astrocytic EAAT-1 (GLAST) and EAAT-2 (GLT-1) transporters, which are responsible for the removal of glutamate from the neuronal synapse (270, 948, 1326).

In crustaceans, for example in the lobster *Homarus americanus* (2062) and the crayfish *Pacifastacus leniusculus* (700), elevated ammonia levels in low-salinity media disrupt ionoregulatory function. Exposure of the green shore crab *Carcinus maenas* to 1 mmol l^{-1} total ammonia leads to increased ion permeability and salt flux across the gill; higher concentrations reduce both variables (1740). In *Penaeus stylirostris*, elevated ammonia levels reduced the total number of immune active haemocytes by about 50% (1022). In the Dungenees crab *Metacarcinus magister*, a two-week exposure to 1 mmol l^{-1} total ammonia causes an increase of hemolymph ammonia to environmental levels, paralleled with a total loss of the branchial capability to actively excrete ammonia and a decrease in branchial mRNA expression levels of a Rh-like ammonia transporter, V-ATPase, Na^+/K^+-ATPase and a cation/H^+ exchanger, all transporters suggested to be involved in ammonia transport processes (1174, 1953). Most literature dealing with ammonia toxicity in non-mammalian systems can be found for fish. Several review articles on this subject have been recently published, such as by Eddy (472), Ip and others (831, 832) and Randall and Tsui (1507, 1874). Among other effects, it was found that increased water ammonia levels decreased critical swimming velocity in rainbow trout and coho salmon (1210, 1667, 1986) and impaired the performance of the "fast-start escape response" in the golden gray mullet *Liza aurata* L. (1209). Further, branchial gas exchange and oxidative metabolism are disturbed by excess ammonia (1997). Based on the studies by Alabaster and Lloyd (15) and on data gained on Atlantic salmon (*Salmo salar*) (16), it was speculated that a reduction in the level of dissolved oxygen in the water increases the toxicity of ammonia to fish (472).

Due to its toxicity, in most species, including mammals (337), fish (666) and aquatic crabs (242, 1945), the ammonia concentration of the body fluids is typically low (50-400 µmol·l^{-1}). Concentrations exceeding 1 mmol·l^{-1} total ammonia (NH_3 + NH_4^+) are usually toxic to mammalian cells (808). In aquatic crustaceans, environmental exposure to ammonia is lethal at relatively low doses. For instance, LC50 after 96 hours of exposure was determined in the crayfish *Orconectes nais* at 186 µmol·l^{-1} NH_3 (720), in the Sao Paulo shrimp *Penaeus paulensis* at 19 µmol·l^{-1} NH_3 and 0.307 mmol·l^{-1} total ammonia (1386) and in the redtail prawn *Penaeus penicillatus* 58 µmol·l^{-1} NH_3 and 1.39 mmol·l^{-1} total ammonia (279). In contrast, terrestrial crustaceans such as *Porcellio scaber* (2036) or *Cardisoma carnifex* (2022) exhibit a much higher tolerance to elevated hemolymph ammonia levels.

Nitrogen Transporters

Ammonia transporting proteins and mechanisms

Ammonia occurs in a pH-dependent equilibrium either as gaseous, uncharged NH_3 or in its ionic form NH_4^+. Due to it relatively high pK (9.2-9.8), the vast amount of ammonia exists in physiological solutions as NH_4^+, although small amounts of NH_3 will always be present. It has long been thought that transepithelial ammonia transport occurs mainly by membrane diffusion of uncharged NH_3 driven only by an existing or generated partial pressure gradient (P_{NH3}). An excretion mechanism solely based on NH_3 membrane diffusion is not likely, however, because membrane permeability of NH_3 is much lower than that of CO_2 (950). Indeed, some plasma membranes of animal epithelia are relatively impermeable to NH_3 as shown for frog oocytes (221), the renal proximal straight tubules (591) and mammalian colonic crypt cells (1697). Therefore, in addition to limited NH_3 membrane diffusion and NH_4^+ crossing epithelia via paracellular routes, for transmembrane movements of ammonia transport proteins are required.

Na^+/K^+-ATPase Perhaps the most important transporter in ammonia-excreting epithelia is the basolateral Na^+/K^+-ATPase, which transports NH_4^+ actively from the body fluids into the cytoplasma of epithelia cells. Soon after Jens Skou discovered the "K-pump" in leg nerves of the green shore crab *Carcinus maenas*, he demonstrated that this pump also accepts NH_4^+ as a substrate, replacing K^+ ions (1708) (Fig. 34A-1).

Masui et al. (1181) showed that the branchial Na^+/K^+-ATPase from the crab *Callinectes danae* is synergistically stimulated by NH_4^+ and K^+, increasing its catalytic activity by up to 90%. The authors came to the conclusion that the two ions bind to different sites of the branchial Na^+/K^+-ATPase. This observation was also attributed to the branchial Na^+/K^+-ATPase of the freshwater shrimp *Macobrachium olfersii* by Furriel et al. (569), who suggested that for this species, at high NH_4^+ concentrations the pump exposes a new binding site for NH_4^+ that, after binding to NH_4^+, modulates the activity of the Na^+/K^+-ATPase independently of K^+ ions. In osmoregulating blue swimmer crabs *Portunus pelagicus*, ammonia activates branchial Na^+/K^+-ATPase but without interactive effects of NH_4^+ and K^+ (1571). In the gills of *Carcinus maenas* acclimated to brackish water, both gradient-driven (1106) and active ammonia excretion (1946) are partially inhibited by ouabain. Further, Mangum and others demonstrated that ouabain inhibits ammonia excretion in the lamellibranch mollusc *Ragia cuneata* and the polychetous annelids *Nereis succinea* and *Nereis virens* by ~40%, 30% and 60%, respectively (1149). Similar observations were made in the freshwater flatworm *Schmidtea mediterranea*. Here ouabain reduced the excretion of total ammonia by approximately 50% (1947). Also for vertebrates it was shown that the Na^+/K^+-ATPase is directly involved

in ammonia transport processes. In the seawater fish *Opasus beta* and *Takifugu rubripes*, branchial enzyme activity was similar when K^+ was replaced by NH_4^+ ions (813, 1147). Further, when exposed to high environmental ammonia (HEA) concentrations, ouabain inhibited active ammonia excretion in the mudskipper *Periophthalmodon schlosseri* (1508). Studies in the mammalian kidney confirmed Na^+/K^+-ATPase mediated transport of ammonia, for example in the inner medullay collecting duct (1925), or proximal tubule (590, 983).

H^+/K^+-ATPase Functional heterologous expression studies in *Xenopus* oocytes revealed that ammonia can also be transported by the apical localized colonic H^+/K^+-ATPase (cH^+/K^+-ATPase), with NH_4^+ substitute for K^+ ions (133, 340, 1794) (Fig. 34A-5). In addition, a study by Swarts and others showed that the enzyme is more active in the presence of NH_4^+ ions than in the presence of K^+ ions (1794). The cH^+/K^+-ATPase is therefore potentially involved in active ammonia transport to protect and counteract mucosal to serosal ammonia influxes in the mammalian colon where extremely high ammonia concentrations can be found due to protein degradation by intestinal bacteria (1118).

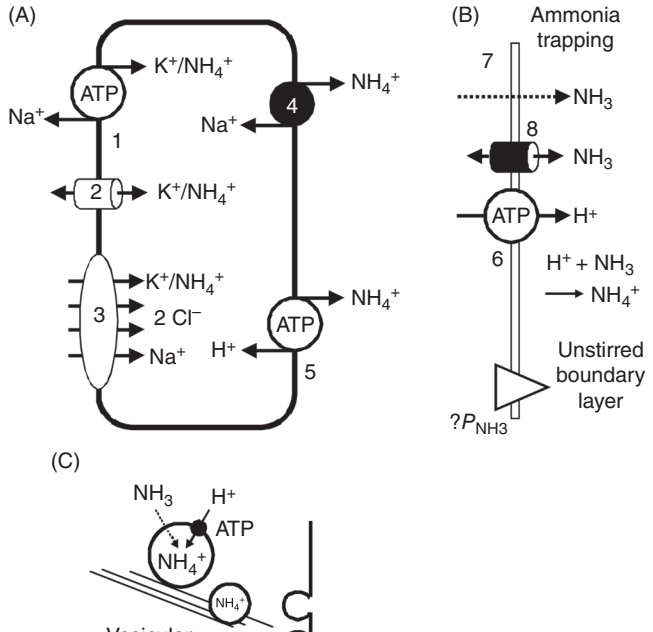

Figure 34 Ammonia-transporting proteins and ammonia-transporting mechanisms. Transporters and putative mechanisms are explained in the text. (A) Fictive cell carrying basolateral Na^+/K^+-ATPase (1), apical or basolateral K^+ channels (2), apical or basolateral NKCC-cotransporter (3), apical or basolateral cation/H^+ exchanger (4), apical H^+/K^+-ATPase. (B) Ammonia-trapping mechanism is energized by H^+-ATPase (6). The resulting ΔP_{NH3} is driving NH_3 membrane diffusion (7) or facilitated NH_3 diffusion via Rh-proteins, Amts or MEPs (8). (C) Vesicular ammonia transport based on ammonia trapping and vesicular NH_4^+ transport to target membrane.

NKCC Besides directly transporting ammonia, the Na^+/K^+-ATPase generates a low intracellular Na^+ concentration, which drives secondary active ammonia transports, for instance by the Na^+-K^+-$2Cl^-$ cotransporter (NKCC) and Na^+/H^+-exchanger (NHE). Using the inhibitor furosemide Good and others demonstrated that the apical NKCC in the mammalian medullary thick ascending limb (MTAL) recognizes NH_4^+ as a substrate (625) (Fig. 34A-3). Also, experiments employing rabbit membrane vesicles from MTAL confirmed that NH_4^+ is accepted by the binding K^+ site of the furosemide- and bumetanide-sensitive NKCC with an affinity similar to that for K^+ (927). In T84 cells, a human intestinal carcinoma cell line, flux studies showed that the basolateral localized NKCC1 mediates blood to cell ammonia uptake (2032).

In fish, studies on the perfused dogfish pup (*Squalus acanthias*) suggest that a portion (~17%) of branchial ammonia excretion is mediated by the basolateral-situated bumetainide-sensitive NKCC (511). Further, sustained elevated expression levels of branchial NKCC1 in the pufferfish *T. rubripes* in response to elevated environmental ammonia indicate the importance of the cotransporter in fish ammonia excretion.

Cation/H^+ exchanger (NHE) (Fig. 34A-4) Also driven by the Na^+/K^+-ATPase are Na^+-dependent cation exchanger (NHEs). Early studies on crustaceans suggested the participation of apical localized cation/H^+ exchangers (NHEs) in ammonia excretory mechanisms. These suggestions were based on the inhibitory effects of amiloride, a rather unspecific blocker for Na^+ channels and at higher dosage also for NHEs (89). Hunter and Kirschner (815) reported a substantial reduction in ammonia excretion of approximately 32% and 56% in the marine osmoconforming crabs *Cancer antenarius* and *Petrolisthes cinctipes*, respectively, when the exchanger is blocked. Gill perfusion experiments in the green shore crab *C. maenas* (1106, 1946) and *C. pagurus* (1945) showed similar results. However, results obtained by using amiloride on epithelia or animals with a cuticle must be reviewed with caution. Experiments employing the isolated cuticle from *Carcinus maenas* have shown that cuticular Na^+ and NH_4^+ conductances (G_{cut}) are inhibited by apically applied amiloride in a dose-dependent manner, with an inhibitor constant $K^{ami\text{-}Na+} = 0.6 \cdot \mu mol \cdot l^{-1}$ for sodium ions and $K^{ami\text{-}NH4+} = 20.4 \cdot \mu mol \cdot l^{-1}$ for ammonium ions, respectively (1375, 1954). Further evidence of the participation of cation-proton exchangers in ammonia transport processes were found in the midgut of the tobacco hornworm *Manduca sexta.* Here luminal-applied amiloride blocked the active ammonia uptake in a dose-dependent manner, suggesting an ammonia transport by a so far non-characterized cation/NH_4^+ exchanger (144, 1943).

In mammals, NHE-3 has been implicated to play a role in renal ammonia transport (1270, 1957). Accordingly it was demonstrated that proximal tubule brush border vesicles exhibit Na^+/ NH_4^+ exchange activity (928) (Fig. 34A-4).

K^+ channels Potassium ion channels are putative candidates to mediate transmembrane NH_4^+ transport. K^+ and NH_4^+ ions have nearly identical biophysical characteristics (1957) and NH_4^+ potentially substitutes the potassium ion at the K^+-binding site of K^+-channels (Fig. 34A-2). As summarized by Choe and others, transport of NH_4^+ by K^+-channels has been shown for various K^+-channel families, including Ca^{2+}-activated, weak and strong inward-rectifying, delayed rectifiers, voltage-gated, and L-type transient K^+ channels (286). The relative conductance of K^+ channels for ammonium ions is approximately 10-20% compared to the conductance of K^+ (286). Using the competitive K^+ channel blocker barium (Ba^{2+}), an inhibition of the ammonia excretion in the mammalian proximal tubule was observed (1695). Also in invertebrates K^+ channels seem to be involved in ammonia transport. Studies employing isolated perfused gills of the green shore crab *C. maenas* showed that inhibition of basolateral K^+-channels by Cs^+ partially blocked both gradient driven and active ammonia excretion (1946). In contrast, in the midgut of the tobacco hornworm *M. sexta* basolateral application of Ba^{2+} showed no effect on the active ammonia uptake, although a ~40% inhibition of the parallel-measured potassium-dependent short-circuit current (I_{SC}) indicated Ba^{2+}-sensitive K^+ channels to be present (1943).

Aquaporins The protein family of aquaporins facilitate first and foremost the transmembrane transport of water (949). However, for some members of this family, permeability for NH_3 also has been demonstrated. Studies on AQP1 employing a *Xenopus* oocyte expression system suggested that AQP1 mediates an increased membrane permeability for NH_3 (1294). AQP1 function as a NH_3 transporter, however, was not confirmed by other investigators employing *Xenopus* oocytes and yeast complementation assays (788, 836). In contrast, ammonia-mediating capabilities were confirmed for four other members of the aquaporin family, namely AQP3, AQP7, AQP8 and AQP9 (1076). All of these transporters increase, when expressed in *Xenopus* oocytes, the membrane permeability for NH_3 but also for the traceable ammonia analog methylammonia (MA) (788, 1603). In AQP8-knockout mice, minor changes of hepatic ammonia accumulation, renal excretion of infused ammonia and intrarenal ammonia concentrations were reported (2052). For a recent review on ammonia-transporting aquaporins and their tissue distribution in mammals, see Litman et al. (1076).

Rh-glycoproteins and Amts In 1994, Marini and others discovered that methylammonium/ammonium permeases (MEPs) are functioning as ammonium transporters (1156). In 2000, the same group demonstrated that the Rh (Rhesus) proteins that were expressed in red blood cells and other tissues in humans also transport ammonia and are analogs of MEP and Amt proteins (1155). A thorough analyses of the Amt/MEP/Rh family of proteins has been conducted, for example, by Huang and Peng and Kitano and others (809, 944). In bacteria and plants, "ammonium transporters" are abbreviated Amts, whereas in yeast they are termed MEPs (methylammonium permeases). As pointed out by Huang and Peng, Rh proteins are phylogenetically related to Amt's with ~14% identity between the amino acid sequences (809). The ammonia-transporting proteins RhAG, RhBG and RhCG (for non-human species Rhag, Rhbg and Rhcg) are glycosylated and part of a group of Rh-50 proteins ("50" for the approximate molecular weight in kDa). Although there are more members belonging to the Rhesus protein family (e.g., Rh30), ammonia transport capabilities have been shown only for the glycosylated forms so far. The debate regarding transport specificity of members of the Amt/MEP/Rh family, however, is still ongoing. Whereas structure analysis and biochemical assays of purified and reconstituted AmtB transporter strongly suggest that the gaseous form NH_3 is transported (915, 916), functional expression studies of vertebrate Rh-proteins differ and are not clear-cut as to the exact molecular species NH_3 or NH_4^+ that is transported. When expressed in HeLa cells, human erythroid RhAG appears to transport both species (88). It was further shown that RhAG transport the ammonia analog methylammonia (MA), and that this transport is pH dependent and electroneutral (1968). RhAG expression in humans is mainly in red blood cells and erythropoeitic tissues, whereas the non-erythroid Rh proteins are expressed in other tissues (e.g., RhBG in kidney, liver, GI tract, ovary and skin, and RhCG in kidney, central nervous system, GI-tract, skeletal muscles and testes [693, 1083, 1084, 1956]). In mammals, RhBG/Rhbg is localized to the basolateral membrane of epithelia cells (1494, 1909). For this transporter as well, it is not clear which species of ammonia, NH_3 or NH_4^+, is transported. Some studies suggest that RhBG/bg mediates an electroneutral NH_4^+/H^+ transport (1111, 1292), whereas other investigations point to a transport of the uncharged form NH_3 (1111, 1145, 2075). RhCG/Rhcg is mostly localized apically, but not as strictly as RhBG/Rhbg is localized to the basolateral membrane. Whereas Rhcg is found exclusively in apical membranes in the mouse kidney (487, 1909), RhCG shows both apical and basolateral expression in the human kidney (691). Also the transport specificity for RhCG/Rhcg is ambiguous and ranges from a transport that mediates both NH_3 and NH_4^+ (62) to an electroneutral transport of NH_3, likely by H^+/NH_4^+ exchange (1111). Most recently the X-ray crystallographic structure of human RhCG was revealed, showing that RhCG forms a trimeric complex and promotes the passage of NH_3. Transport of NH_4^+ was not precluded, although the crystallographic structure of RHCG predicts the exclusion of NH_4^+ transport due to the protein's hydrophobic lumen (672). Mammalian Rh-proteins have been reviewed extensively (1958) but molecular evidence for several other Rh-protein related proteins has also been found in non-mammalian species. A phylogenetic genetic analysis revealed that Rhag, Rhbg and Rhcg are exclusively expressed in vertebrate species, whereas RhP1/RhP2 are predicted to be phylogenetically older and are found in invertebrates (809).

In teleost fish so far, five Rh-glycoproteins have been identified: Rhag and two subforms of Rhbg (Rhbg1, Rhbg2) and Rhcg (Rhcg1, Rhcg2) (1289, 1297). Expression for Rhag was found in red blood cells (RBC), spleen and kidney and in the membranes of branchial pillar cells (1289). Rhbg was found to be expressed in multiple tissues including RBC, brain, eye, heart muscle, intestine, kidney, liver skeletal muscle, skin, spleen and the gill epithelium (814, 1297). A study in pufferfish (*Takifugu rubripes*) gills revealed that Rhbg in fish is also localized to the basolateral membrane (1289). At least in *T. rubripes* Rhcg1 is localized to the apical membrane of MR cells at the base of the gill lamellae, whereas Rhcg2 is localized to the apical membrane of branchial pavement cells (1289). A functional expression study in *Xenopus* oocytes employing Rh-proteins cloned from trout gill and using the ammonia analog MA showed that all five fish ammonia transporters mediate ammonia transport. That ammonia is indeed transported by pufferfish Rhag and Rhcg2 was confirmed by using NH_4^+-selective microelectrodes (1300). The importance of Rh-proteins was also shown in the developing zebrafish *Danio rerio*. Morpholino knockdown experiments revealed a ~50% reduction of total ammonia excretion in the fish upon silencing of each individual transporter including Rhag, Rhbg and Rhcg2 (191). In elasmobranchs, expression of only one Rh-isoform (Rhbg) was confirmed. In the little skate *Leucoraja erinacea*, Rhbg mRNA was found in low volumes in intestinal sections, but at high expression levels in the rectal gland and kidney (34). Most recently Nakada et al. discovered methylammonia transport capability for Rhp2, another glycolsylated protein belonging to the Rh-like ammonia transporter family found in the banded hound shark, *Triakis scyllium.* This protein appears to be expressed in the second and fourth loop of the sinus zone of the elasmobranch's kidney, where it is localized to the basolateral, interstitium facing membrane. The function of Rhp2 is unclear, although it may be involved in renal ammonia reabsorption (1290).

Besides the studies of fish noted above, mRNA expression of Rh proteins was also confirmed in the ammonia-transporting gills and pleopods of a variety of different haline crustaceans, among them the stenohaline marine crab *Cancer irroratus* (GenBank accession: AY094179), the euryhaline crabs *C. sapidus* (GenBank accession: AY094178) and *C. maenas* (1950), the isopod *Idotea baltica* (GenBank accession: AY094181) and the true freshwater crab *Dilocarcinus pagei* (GenBank accession: AY094180). So far in crustaceans only one Rh isoform has been identified. In the tobacco hornworm *Manduca sexta*, mRNA expression analysis revealed a moderate abundance of an Rh-like protein in the midgut, trachea and fatbody and high expression levels in the hindgut, ganglia and the Malpighian tubules (1943). The cellular localization of arthropod Rh-like ammonia transporters has not been determined yet (Fig. 34B-8). In addition to findings in the tobacco hornworm, Rh-proteins have been identified in other insects as well. In the yellow fever mosquito *Aedes aegypti*, two Rh-isoforms have been identified, Rh50-1 and Rh50-2. Both are expressed in the ammonia-excreting anal papillae of the freshwater-inhabiting larvae and respond to increased environmental ammonia levels (1948).

Interestingly, in insects and other invertebrates, for example *C. elegans*, a genome analysis revealed the presence of Amt transporters also. Although the function of the Amts in animal systems as ammonia-transporting proteins have not been investigated yet, these Amts group together with functional Amts identified in plants (1948) and critical amino acids in the pore center (H168 and H318 in *E. coli* AmtB) are conserved, at least in the insect Amts.

The gene expression data base for *Drosophila melanogaster* (FlyAtlas [285]) indicated that the Rh-protein and Amt of the fly are expressed in the same tissues, however, in most cases with complementary mRNA expression levels. While Rh50 in the fruit fly *D. melanogaster* was highly expressed in neuronal tissues, Malpighian tubules and hindgut, mRNA levels of Amt were low in those tissues. In contrast, in the salivary glands, Amts showed increased expression levels, while low expression levels were detected for Rh50.

In-situ hybridization studies in the freshwater planarian *S. mediterranea* indicated that an identified Rh-like ammonia transporter is strongly expressed in the epidermal epithelium. Together with responses of mRNA expression levels of this transporter upon changes of environmental ammonia or pH (1947), this suggests a role of the epidermal Rh-protein in ammonia excretion processes.

V-type H^+-ATPase and ammonia trapping Although not capable of transporting ammonia by itself, the V-type H^+-ATPase plays a crucial role in transepithelial ammonia excretion and uptake processes. Membrane diffusion of NH_3 strictly depends on the transmembrane NH_3 partial pressure gradient (ΔP_{NH3}), often generated by the action of the V-ATPase or cation/H^+ exchanger. The action of both transporters is responsible for lowering locally the pH on one side of the membrane and generating thereby a transmembrane ΔP_{NH3}, a mechanism referred as "ammonia trapping" (Fig. 34B). The transport of ammonia via Rh glycoproteins can also be energized by the proton pump, regardless of whether the Rh-proteins function as NH_3 channels or H^+/NH_4^+ exchangers. In freshwater fish, the importance of the V-ATPase in ammonia excretion processes has been described extensively (e.g., 191, 1665, 1953, 2007, 2041). For example, eliminating the low pH in the apical unstirred gill boundary layer by setting the pH to 8 using HEPES caused a significant reduction in ammonia efflux rates in the rainbow trout *Oncorhynchus mykiss* (2007). In zebrafish (*Danio reio*), larval ammonia extrusion via the skin depends directly on the presence and action of a H^+-ATPase, as demonstrated by reduced ammonia excretion rates in H^+-ATPase knockdown animals and the inhibitory effect of bafilomycin A1, a specific inhibitor for the V-Type H^+-ATPase (163, 1665).

Also in invertebrates, a V-type H^+-ATPase linked to ammonia transport has been demonstrated. In the green shore crab *C. maenas*, branchial active ammonia excretion was reduced by 66% after blocking the proton pump with bafilomycin A1. It is noteworthy that, in contrast to freshwater fish, the H^+-ATPase in the crab gill epithelium is localized to vesicles rather than the apical membrane (1954). Further, the active ammonia uptake in the midgut of the tobacco hornworm *M. sexta* was significantly reduced after bafilomycin A1 application, indicating participation of a V-ATPase in the intestinal ammonia uptake mechanism (1943).

In the kidney of rats, expression of the apical ammonia transporter Rhcg seems to be colocalized with the presence of an apical H^+-ATPase. In cortical collecting ducts, H^+-ATPase-rich intercalated cells showed parallel apical Rhcg abundance, whereas H^+-ATPase-negative principal cells showed only a faint Rhcg expression. In the outer medulla and the upper portion of the inner medulla, Rhcg abundance was limited to a subpopulation of cells within the collecting duct with an apical H^+-ATPase, indicating that Rhcg expression in medullary collecting ducts is linked to the action of an apical proton pump (487).

Vesicular ammonia transport In the gills of the green shore crab *C. maenas*, gradient-driven or active ammonia excretion was substantially inhibited (~60-100%, depending on the experiment and drug) after the application of microtubule inhibitors such as colchicine, taxol and thiabendazol (1954). This finding led to the suggestion of a vesicular ammonia-trapping mechanism in which cellular NH_3 moves via membrane diffusion or through Rh-proteins into acidified vesicles to be transformed into its membrane-impermeable ionic form, NH_4^+. For directed excretion these NH_4^+-loaded vesicles would then be transported to the apical membrane for exocytotic release. A similar intracellular ammonia transport, which depends on a vesicular H^+ ATPase and an intact microtubule network, was also described for the active ammonia uptake in the midgut epithelium of the terrestrial tobacco hornworm *Manduca sexta* (1943) (Fig. 34C).

Urea Transporting Proteins and Mechanisms

The urea transporters (UT) play a crucial role in urine concentration and urea recycling in the mammalian kidney. All UTs promote a facilitated urea transport (445, 525, 2051). UTs belong to two different subfamilies encoded by two different genes, UT-A and UT-B. Several UTs belong to the UT-A family. UT-A1 is expressed exclusively in IMCD cells and its activity is regulated by vasopressin (AVP). UT-A2 is localized to the lower portions of the thin descending limbs of short loops of Henle, whereas UT-A3 is restricted to the terminal IMCD, where it is localized to both the apical and basolateral membranes. UT-A4 and UT-A5 were detected extrarenally in heart and testis, respectively. UT-B has been found in RBC and many different tissues including the endothelium of the arterial vasa recta (525) and extra-renal tissues like bone marrow, spleen, fetal liver, GI tract, ureter and bladder and astrocytes (2051).

Evidence for urea transporters have also been found in tissues of non-mammalian vertebrates. Examples include the renal tissues of fish (1238) and amphibians (1094, 1628), the ventral skin of toads (213, 468, 478), the gills of teleost fish (1239, 1927, 1928) and the gills, rectal gland, kidney and intestine of elasmobranchs (34).

In the gills of the spiny dogfish, evidence was found for a secondary active Na^+-urea exchanger energized by the basolateral Na^+/K^+-ATPase and localized to the basolateral membrane. This phloretin-sensitive urea transporter serves most likely to retain urea in elasmobranchs where high plasma urea concentrations are maintained for osmotic homeostasis (531). In addition, studies on brush border membrane vesicles from kidney tissues of the little skate *Raja erinacea* revealed evidences for two additional types of UTs: a phloretin-sensitive, non-saturable uniporter in the dorsal section and a phloretin-sensitive, sodium-linked urea cotransporter in the ventral section of the kidney (1249). Sequence information of the expressed UT mRNAs has not been linked yet to the different kind of UTs in elasmobranchs. UT transcripts, termed efUT-1, efUT-2 and efUT-3, have recently been identified in the holochephalan elephant fish, *Callorhinchus milii*. efUT-1 is orthologous to the elasmobranch UTs, whereas efUT-2 and efUT-3 are novel UTs. When functionally expressed in *Xenopus* oocytes, all UT transcripts, including the short and long variants of efUT-1 and efUT-2, induced increases in ^{14}C-urea uptake of more than 10-fold. Phloretin sensitivity of urea uptake suggested that the identified UTs are facilitative transporters (877). The physiology of urea transport in fish has been reviewed extensively by McDonald and others (1202). Information gained from Expressed Sequences Tags (EST), for instance from the blue crab *Callinectes sapidus* (GenBank accession #: CV527852) or the jewels wasps *Nasonia vitripennis* (GenBank accession #: XP_001601729), indicate that urea transporters are also expressed in invertebrate tissues.

Aquaporins

Some mammalian aquaporins, mostly in the subclass of aquaglycoproteins (GLPs), transport organic compounds such as glycerol and urea in addition to water (698, 964). Urea-transporting GLPs have been identified among them, specifically AQP3, AQP7, AQP9 and AQP10. For a recent review on urea-transporting aquaporins, their tissue distribution and co-localization with urea transporters in mammals, see also Litman and others (1076, 2003). For non-mammalian species, urea permeability has not been shown to date, most likely because it has not been investigated yet. Molecular analysis and functional expression studies of three cloned AQP-like proteins (HC-1, HC-2 and HC-3) in the gray tree frog

Hyla chrysoscelis revealed that at least one of them, HC-3, exhibits glycerol transport capabilities and shows a very high degree of nucleotide identity (> 80%) to the human urea-transporting aquaglycoprotein AQP3 (2076). One can predict that the amphibian aquaporin-like protein HC-3 also mediates urea permeability.

Uric Acid Transporting Proteins and Mechanisms

With a pKa1 = 5.75, uric acid behaves as a weak acid and occurs predominantly (~98%) as urate anion at physiological pH. The urate anion cannot readily pass through cell membranes, and for epithelia, excretion transporters are required. By far the most literature on urate transporters can be found on mammals, through to the role of urate transport in diseases like gout (1724). In mammals, nearly all plasma urate is filtered by the renal glomeruli; however, close to 90% of the initial filtrate is reabsorbed in the proximal tubule back into the blood stream. Beside renal excretion, urate is also excreted by hepatocytes into the bile canniculi and into the gastrointestinal tract. A key player for urate reabsorption is the apical localized URAT1, which is proposed to transport intracellular organic anions such as lactate in exchange for urate (496, 1609, 2033). It is speculated that URAT1 is functionally coupled to apical sodium monocarboxylate cotransporters, SMCTI and SMCT2 (827). Mediated by the Na^+/K^+-ATPase-powered, inwardly directed Na^+ gradient, both proteins transport lactate into the cells, which can in turn be utilized by URAT1. Another transporter involved in renal urate reabsorption is the apical localized organic anion transporter-4 (OAT4), which acts as a low-affinity urate (anion)/dicarboxylate exchanger (47, 684). For basolateral exit, three transporters with urate transport capabilities have been proposed: (i) the organic anion transporter-1 (OAT1) (828, 1647); (ii) the organic anion transporter-3 (OAT3), which functions as a urate-decarboxylat exchanger (61); and (iii) possibly the most important transporter, the glucose transporter-9 (GLUT9), which transports urate 45- to 60-fold faster than glucose/fructose (264) and functions as a high-capacity/low-affinity urate transporter (1915). For apical urate excretion, several transporters have been discussed, including a splice variant of GLUT9 (GLUT9ΔN), the sodium phosphate transporters-1 and -4 (NPT1, NPT4), the voltage-driven organic anion transporter, OATv1 and the ATP-binding cassette transporter, MRP4. GLUT9ΔN shows apical expression (54); however, urate transport capacities have not been shown yet. In contrast, for OATv1, NPT1, NPT4 and MRP4 urate transport capability has been demonstrated (48, 1880, 1896). Aside from its apical expression in renal cells, MRP4, which mediates an ATP-dependent urate transport, it is also found basolaterally in liver cells and is therefore suggested to be responsible for hepatic urate transport into the circulatory system (1896). Very little is known about urate transporters in non-mammalian systems. However, genome and EST projects on a wide variety of other systems including amphibians, fish, insects, crustaceans and nematodes will certainly promote future studies regarding this subject.

Nitrogen Excretion of Taxonomic Groups

With some exceptions, aquatic invertebrates, teleost fish and amphibian larvae are ammoniotelic, excreting the majority of their nitrogenous waste products in the form of ammonia. Adult amphibians, mammals and marine elasmobranchs are in general ureotelic, with the main excretory waste product being urea. Most adult insects, arachnids, birds and reptiles are uricotelic, excreting predominately uric acid or other purine-based compounds like guanine, allantoin and xanthine. Often, however, depending on the lifestyle or the particular stage of life history, a single species might switch from one main excretory product to another as seen, for example, in amphibians. For animals, our knowledge of excretory mechanisms of any given nitrogenous waste product is still very limited. So far, information on excretory processes and mechanisms has been gained usually by performing flux studies and studying the effects of transporter-specific inhibitors. However, over the last two decades or so, powerful molecular techniques such as functional gene expression analysis of putative transport molecules and quantitative mRNA, as well as protein expression analysis and immunohistochemistry, also have been employed. The vast majority of these studies, including molecular approaches, have been performed in mammals and fish and were covered extensively in recent years (191, 813, 1112, 1205, 1289, 1468, 1665, 1953, 1959, 2032, 2041). An extensive review on nitrogen excretion in invertebrates has been provided by O'Donnell (1338) and thus will only be updated here.

Protozoa, porifera and cnidaria Studies on nitrogen excretion in free living protozoa, porifera and cnidaria are sparse. The free living freshwater microflagellates, *Monas sp*., exhibit average ammonia excretion rates of 0.76 to 1.23 μmol of NH_4^+ per mg (dry wt) per hr (1664). So far, the mode of ammonia excretion in protozoa is unclear. Freshwater protists, such as *Amoeba proteus*, osmoregulate by means of a contractile vacuole. In the membranes of this contractile vacuole high abundance of a V-type H^+-ATPase was detected (1315). The proton-pumping action of this ATPase will likely generate a cytosol to vacuolar H^+ gradient and thereby also a ΔP_{NH3}, promoting ammonia trapping via membrane diffusion. This NH_3 movement might be supported by a so far unidentified Rh-protein within the same membrane. Therefore, it is conceivable that ammonia waste in freshwater protists is excreted along with excess water by the action of the contractile vacuole. Contractile vacuoles have also been found in freshwater sponges, here in amoebocytes, pinacocytes and choanocytes (181). For the symbiotic sponge *Haliclona cymiformis*, an ammonia excretion rate of 4.6 μg ammonium-N g^{-1} dry mass h^{-1} was described. Interestingly, ammonia released by the sponge serves as an important nitrogen source for its symbiotic partner, the rhodophyte *Ceratodictyon spongiosum* (398). This kind of symbiotic partnership was also suggested for the giant sea anemone *Entacmaea quadricolor* and its symbiotic zooxanthella partner. In fact, at times of

high demand of nitrogenous compounds by the algae during daytime, *E. quadricolor* exhibits an ammonia uptake, likely waste ammonia from the anemoefish *Amphiprion bicinctus*. At nighttime, when metabolic demands of the algae are minimal, the rate of ammonia uptake by the sea anemone decreases to near zero (1575). In contrast, aposymbiotic sea anemones (lacking zooxanthella) *Aiptasia pulchella* excrete ammonia during daytime (1995). Mechanisms of ammonia uptake or excretion in cnidaria are largely unknown. A genome project revealed that the starlet sea anemone *Nematostella vectensis* does likely express a Rhesus-like ammonia transporter, exhibiting ~44% identity in its amino acid sequence to the ammonia-transporting mammalian Rh-glycoproteins. In addition, genes for several putative AMT-like ammonia transporters have been identified in this species.

Nematoda, planaria and annelida

Parasitic nematodes such as *Ascaris lumbricoides* (1960), *Trichinella spiralis* larvae (713) and *Nematodirus* sp. (1569) excrete ammonia as their primary waste product. Similarly, the free-living nematode *Caenorhabditis briggsae* also excretes predominately ammonia but no significant amounts of urea or uric acid (1580). Possible excretion routes include the integument, the nephridial system or the gut. In the genetic model system, *Caenorhabditis elegans* genes for two Rh homologues, CeRh1 and CeRh2, have been identified that are highly conserved and similar to the human Rh-glycoproteins (851). A transgenic analysis using reporter genes showed that CeRh1 is mainly expressed in hypodermal tissue, with lower expression also in other cell types (851). These findings suggest that ammonia is excreted, at least partially, via the integument.

Knock-down experiments revealed that CeRh2 is not essential for embryonic development, while silencing CeRh1 caused a lethal phenotype mainly affecting late stages of *C. elegans* embryonic development (851). In addition to the Rh genes, *C. elegans* also expresses 4 AMT-like ammonia transporters (amt-1 to amt-4; see http://www.wormbase.org.). While no information is available to date for expression patterns of amt-1, amt-2 and amt-4, amt-3 is expressed in adult nematodes in the head nerves, ring and tail nerves and in the intestine. Expression in the nerve tissues might serve protective purposes, while abundance of amt-3 in the intestine might facilitate ammonia excretion or ammonia uptake. However, verification that amt-proteins expressed in animal tissues indeed promote ammonia transport must still be obtained.

So far little is known about nitrogen excretion in planarians. A most recent study on the freshwater planarian *Schmidtea mediterranea* revealed that ammonia is excreted across the mucous epidermis in an active fashion at a rate of 0.70 ± 0.03 μmol^{-1} gFW^{-1} h^{-1} (1947). This excretion appeared to be dependent on the environmental pH and inhibitor experiments combined with gene-expression studies indicated further a number of transporters involved in the ammonia excretion mechanisms that include the V-ATPase, Na^+/K^+-ATPase, cation/H^+ exchangers, the carbonic anhydrase and a Rh-like ammonia transporter. According to a proposed working model (Fig. 35), interstitial fluid ammonia is pumped as NH_4^+ across the basolateral membrane by

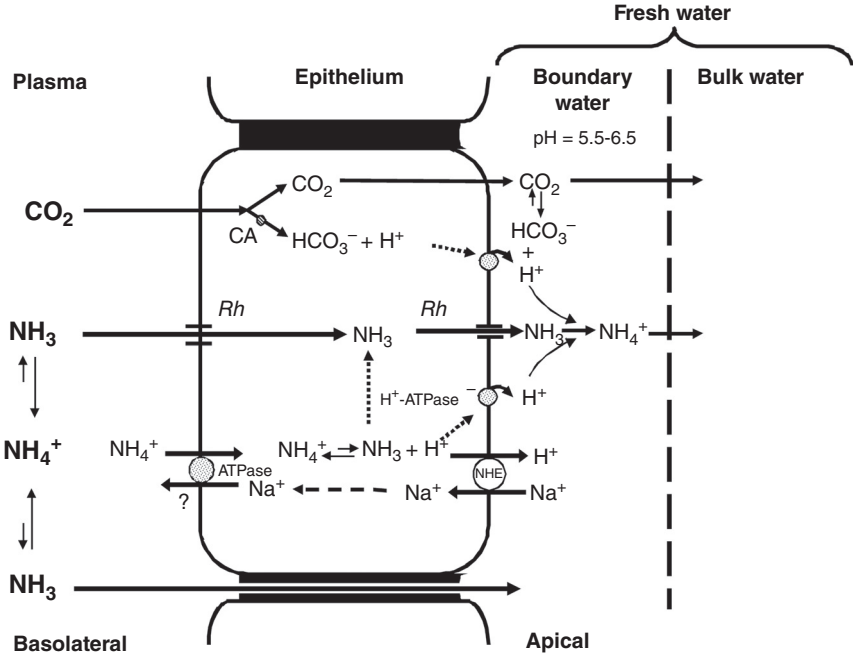

Figure 35 Ammonia excretion across the epidermal epithelium in the freshwater planarian *Schmidtea mediterranea*. The proposed mechanism is described in the text. The figure is modified after (1947).

Na^+/K^+-ATPase into the cytoplasm. Protons provided by a cytoplasmatic carbonic anhydrase are then excreted apically via the V-ATPase and a cation/proton exchanger (NHE), acidifying the unstirred boundary layer to a pH of approximately 5.5-6.5.

The generated low pH protonizes apical NH_3 into its ionic form NH_4^+ and thereby creates a transcellular P_{NH3} gradient. It is proposed that an Rh-like ammonia transporter mediates the NH_3 transport across the apical cell membrane. Due to fact that in freshwater organisms epithelia facing the environment generally show a very low ion conductance to avoid passive effluxes along a steep osmotic gradient (299, 1942, 1945), it is therefore assumed that paracellular NH_4^+ diffusion is negligible. Interestingly, the ammonia excretion mechanism in this flatworm reveals striking similarities to the current model suggested to be in function in gills of freshwater fish (see below).

Animals belonging to the phylum of Annelida are usually ammoniotelic. Ammonia excretion rates of the marine and osmoconforming lugworm *Arenicola marina* were measured as ~33 µmol g dry weight^{-1}day^{-1} (calculated from [1519]). This excretion was salinity dependent, and an acute reduction of the environmental salinity to 16‰ resulted in a strong increase of the excretion rate. After its maximum between 12 hrs and 36 hrs after exposure the excretion rate gradually decreased to levels only slightly above the control rates. This pattern was mirrored in the parallel oxygen consumption rates. Varying ammonia excretion rates were related to amino acid-based extra and intracellular volume regulation in this annelid (1519). Also the freshwater living medicinal leech *Hirudo medicinalis* excretes predominantly ammonia (1870) after ingestion of serum. Excretion rates rapidly increased to a peak of ~30 µmol ammonia g FW^{-1} day^{-1} within a day of feeding, with a second peak starting at day 5-7. After day 15, post-feeding excretion rates dropped slowly to zero over the next week. Interestingly, serum feed *H. medicinalis* also exhibits a peak of urea excretion one day after feeding (~14 µmol urea/ animal/day), which dropped to values less than 2 within the following day. The early ammonia and urea excretion peaks probably result from the elimination of ammonia and urea ingested with the serum, which contained 2.2 mM ammonia and 6.6 mM urea. Ammonia excretion rates of the second phase are very likely due to the digestion of serum proteins. Symbiotic microorganisms do not significantly contribute to ammonia formation (1870). Not much is known about ammonia excretion mechanisms in Annelida. The polychetous annelids *Nereis succinea* and *Nereis virens* actively excrete ammonia. Active ammonia excretion by the polychetous annelids *N. succinea* and *N. virens* depends partly on the activity of the Na^+/K^+-ATPase and can be inhibited with ouabain by 30% and 60%, respectively (1149). The medicinal leech *H. medicinalis* osmoregulates via the integument, exhibiting an active amiloride sensitive Na^+ uptake that is driven by the Na^+/K^+-ATPase (1940, 1941). The skin is further coated with a mucus layer, providing an apical unstirred boundary layer (306). It is conceivable that ammonia excretion occurs via the integument by utilizing transporters involved in osmoregulatory ion uptake. Additional excretory pathways via the metanephridial system or the intestinal tract, e.g. the Na^+/K^+-ATPase energized caecum (1222), might play also a role. Other possible tissues for ammonia elimination in annelids are the well-vascularized parapodia of marine polychaetes as pointed out by O'Donnell (1338). Due to their primary function in locomotion, the tissue is well ventilated and therefore also used for respiratory gas exchange. Consequently, the transepithelial hemolymph to environment ammonia gradient is very likely favorable for ammonia excretion in animals inhabiting the free water column (1338). The terrestrial living earthworm *Lumbricus terrestris*, an oligochaete, excretes predominantly ammonia while feeding and water supply is not limited. It becomes ureotelic, however, when fasted or when water supply is limited (1117). Ammonia excretion occurs here likely via the gut, where extremely high ammonia concentrations (11-104 mM) can be detected (1830). Similar to *H. medicinalis,* an amiloride-sensitive Na^+ transport across the skin was documented in *L. terrestris* as well (979). Therefore, the possibility of NH_4^+ excretion via the skin cannot be excluded. In contrast to the pathways of ammonia excretion, urea is eliminated via the metanephridial system (1830). It is noteworthy that in *L. terrestris* all enzymes for a functional urea cycle have been identified (140).

Ecinodermata

Echinoderms are ammoniotelic. After collection from the sea, and assumed to have recently fed, the sea urchin *Tripneustes gratilla* excretes ammonia at rates of ca. 0.5 µmol g dry weight h-1. This rate is similar to rates found in collected horned sea stars *Protoreaster nodosus* and the brittle star *Ophiorachna incrassate*. Ammonia excretion in these three different echinoderms seems to depend on the time of the day. While oxygen consumption rates remained similar, ammonia excretion rates decreased in animals collected during nighttime (466). Investigations by Sabourin and Stickle on the brackish water tolerant sea cucumber *Eupentacta quinquesemita* and sea urchin *Strongylocentrotus droebachiensis* showed ammonotely for both species in full-strength seawater, with minor excretion of urea (~2-3% of total N-excretion) and primary amines (2-4% of total N-excretion). Apparently nitrogen excretion rates are salinity dependent in sea cucumber but not in sea urchin.

While in *Eupentacta quinquesemita*, ammonia excretion rates are reduced by ~50% when acclimated to 20‰ and 15‰ salinity, excretion rates of primary amines increased approximately 4-fold and 10-fold, respectively. In both species, urea excretion rates remained low and did not changed in response to changing salinities (1592). One can speculate that in low salinity, primary amine synthesis is enhanced for the purpose of intracellular volume regulation and that increased excretion/loss of these energetically valuable molecules is

rather a negative side effect of the salinity stress handling than for the purpose of nitrogen excretion per se. A genome project, supported also by expressed sequence tags, on the California purple sea urchin, *Strongylocentrotus purpuratus* indicates that echinoderms also express Rh-ammonia transporters (GenBank accession #: XP_789738, XM_784645) and an urea transporter-like protein (*GenBank accession #:* XM_781359). To date it is not known in which tissues the Rh-protein and urea transporter are expressed, or their specific role in ammonia and urea excretion.

Mollusca

Cephalopods are quite active compared to others molluscs. These carnivorous animals excrete the majority of their nitrogenous waste as ammonia, with the ctenidia playing a significant role in excretion (1473). Pelagic squid, such as *Illex illecebrosus* are in general more active than bentic octopuses and excretion rates in these animals are high (1.4 mM NH_4^+/kg/hr) at rest but increase about fivefold during exercise (776). Similar excretion rates were also reported for *Loligo forbesi*, an active nektonic predator (158). In contrast, ammonia excretion rates in *Octopus rubescens* were about five times lower (at rest) when compared to plelagic squid *I. illecebrosus* (776).

In addition to ammonia, both squid and octopus also excrete considerable amounts of their nitrogenous waste in form of urea. For instance, at rest, urea accounts for 24% and 30% of total nitrogen excretion in the squid *Illex illecebrosus* and *Octopus rubescens*, respectively (776). The mode of ctenidial ammonia excretion is so far unknown, but ammonia excretion via the kidney is thought to occur by acid trapping, as reported for *Octopus dolfeini* (1473). The pericardial fluid in this octopus is slightly alkanline and contains lower total ammonia concentrations than does the blood (1473). In addition to excretion, in squid, ammonia is also used for buoyancy. These ammoniacal animals sequester ionic NH_4^+, which is lighter than, for example, Na^+, Ca^{2+}, Mg^{2+} or SO_4^{2-}, either in specialized "coelomic chambers," observed in squid of the family Cranchiidae, or in the muscle tissue (other ammoniacal squid). Here the mantle and arms are interspersed with vacuoles that most likely contain ammonium. Ammonium concentrations in these specialized organelles are very high, ranging between 300 and 500 mmol l^{-1} (302, 2040). Considering the toxicity of ammonia, it is noteworthy that in ammoniacal squids the body mass is comprised of approximately 50-60% ammonium fluid (302).

Lamellibranchs are also ammoniotelic. Interestingly, they also excrete considerable amounts of their nitrogenous ballast in the form of amino acids. For instance, sand-mussels *Donax serra* and *D. soriddus* excrete 70% and 73% of their total dissolved nitrogen as ammonia and 30% and 22% in the form of amino acids, respectively (325). Since bivalves do not possess biochemical pathways to detoxify ammonia into less toxic urea or uric acid (141), the excretion of these nitrogenous compounds is negligible, with excretion rates of less then 1% of total nitrogen release (325). In contrast to marine species, freshwater bivales are sometimes challenged by extended periods of air exposure during droughts of unpredictable lengths. A study on the freshwater clam *Corbicula fluminea* showed that during air exposure, hemolymph ammonia levels remain low at pre-emersion levels (60 to 140 μM) for about 4 days before they dramatically increase over the next 3 days to 8 mM, resulting in death of the animal. The data suggest that *Corbicula fluminea* as a representative of freshwater bivalves appear to be able to suppress protein catabolism during the first few days of emergence to avoid toxic ammonia accumulation (237). Not much is known about the ammonia excretion mechanism in bivalves. A study by Mangum and others in euryhaline *Ragia cuneata* suggests that ammonia is actively excreted. Inhibitory effects on ammonia excretion rates employing oubain suggest the participation of the Na^+/K^+-ATPase in this process, as shown for other aquatic invertebrates as well (1149, 1946). Similar to the lamellibranchs, marine prosobranch snails also do not excrete urea (457). In *Thais* (*Nucella*) *lapillus*, ammonia excretion was found to be salinity dependent, with significantly lower rates detected in low salinity (17.5 ppt salinity) when compared to higher salinities (1763). Regardless of the environmental salinity and temperature, these snails excrete/lose also primary amines. However, when expressed as a percentage of ammonia excretion rates, in low-salinity environments the loss of primary amines is higher than the loss in higher salinities (1763). Intertidal polyplacophora such as *Chiton pelliserpentis* are also ammonotelic; they do, however, excrete high amounts of urea. In high shore *Chitons*, which presumably are exposed to air for prolonged time periods, urea-N can make up to 34% of combined ammonia and urea nitrogen, whereas urea-N excretion rates in low shore *Chitons* are lower (798). In contrast to their acquatic relatives, terrestrial snails are uricotelic/purinotelic, excreting their nitrogenous waste predominantly as uric acid, guanine and xanthine (458, 850, 1854). For instance, in the kidney contents (excreta) of the giant South American snail *Strophocheilus oblongugus*, 70% of total N accounted for purinic compounds, with uric acid being the most prominent (~64%), followed by xantine (~24%) and guanine (12%) (1854). In the garden snail *Helix pomatia*, almost all (90%) of the nitrogen is excreted in the form of purines (850). In addition to purinic compounds, shelled snails also excrete gaseous NH_3 through the shell at rates between 1.0 μmol and 0.01 μmol NH_3/g live wt/day. Shell-less slugs, in contrast, absorb NH_3 from the atmosphere (1090).

Crustacea

The main site for ammonia excretion by aquatic crabs is the phyllobranchiate gill (307, 963, 1516), featuring a single-cell layered epithelium covered by an ion-selective cuticle (56, 1049, 1380, 1954). Several transporters and enzymes putatively linked and involved in ammonia transport have been shown to be present in the branchial epithelium of crabs,

among them the Na^+/K^+-ATPase, cation/H^+-exchanger, K^+-channels, Na^+-K^+-$2Cl^-$ cotransporter, H^+-ATPase and the Rh-proteins (1557, 1848, 1950).

Ammonia excretion in aquatic crabs Studies on isolated perfused gills of several aquatic crabs showed that ammonia can be excreted actively against a four- to eightfold inwardly directed ammonia gradient across both anterior and posterior gills to a similar degree, despite their different morphological and physiological characteristics (339, 629, 1848, 1945). Under physiologically relevant conditions, the potential for active branchial ammonia excretion is significantly greater in the marine *Cancer pagurus* than in freshwater-acclimated Chinese mitten crabs *Eriocheir sinensis.* This is remarkable since *Cancer pagurus* gills have a much larger ionic conductance (~250-280 $mS \cdot cm^{-2}$) compared to that of *Eriocheir sinensis* gills (~4 $mS \cdot cm^{-2}$). It is noteworthy that posterior gills of *Carcinus maenas*, thought to play the dominant role in osmoregulatory NaCl uptake, and also anterior gills, thought to be primarily responsible for gas exchange, are equally capable of active ammonia excretion (1945).

In the blue crab *Callinectes sapidus,* ammonia excretion rates are correlated with Na^+ absorption (1482). The same result was obtained for both the Chinese crab *Eriocheir sinensis* (1419) and the shore crab *Carcinus maenas* (1106). In the marine crab *Cancer pagurus*, active branchial excretion of ammonia is completely inhibited by ouabain (1945), suggesting the Na^+/K^+-ATPase is the only driving force for excretion. However, in the gills of *Carcinus maenas* acclimated to brackish water, both gradient-driven (1106) and active ammonia excretion (1946) are only partially inhibited by ouabain, indicating a second active mechanism responsible for branchial ammonia extrusion in this species.

The presence of an apically located amiloride-sensitive Na^+/NH_4^+ exchanger, transporting NH_4^+ from the epithelial cell into the ambient medium in exchange for Na^+, has been suggested for *Callinectes sapidus* (1482) and for *Carcinus maenas* (1106, 1688). Indeed, branchial mRNA expression of a Na^+/H^+-antiporter, putatively transporting NH_4^+ ions as well, was demonstrated in *Carcinus maenas* (1847) and in *Eriocheir sinensis* (1952).

Further studies on the branchial ammonia excretion mechanism in *Carcinus maenas* employing the K^+-channel blocker Cs^+ revealed that basolateral but not apical K^+-channels play a role in the excretory process (1946). In addition, experiments inhibiting the branchial V-Type H^+-ATPase by bafilomycin A_1 resulted in a reduction of active ammonia transport by 66%, identifying the H^+-ATPase as an additional active component in the excretory mechanism of the shore crab (1954). While in *Eriocheir sinensis* a V-Type H^+-ATPase has been localized to the apical membrane of the gill epithelium (1379), in *Carcinus maenas* this pump was found predominantly in the cytoplasm, likely associated with vesicles (1955). This latter finding led to the suggestion (1954) of a vesicular ammonia-trapping mechanism in which cellular NH_3 diffuses into acidified vesicles to be transformed into its membrane-impermeable ionic form NH_4^+. For a directed excretion, these NH_4^+-loaded vesicles would then be transported to the apical membrane for exocytotic release. Such an excretion mechanism was supported by data showing total inhibition of active ammonia excretion by blockers of the microtubule network, including colchicine, thiabendazole and taxol (1954), and the fact that buffering of the experimental solutions by 2.5 mM TRIS (pH 7.8) had no effect on the active ammonia excretion rates. This indicates that acidification of the subcuticular space or the gill boundary layer has no influence on driving ammonia across the apical membrane of the gill epithelia (1945, 1946). The resulting hypothetical model of the ammonia excretion in *Carcinus maenas* is described in detail in Fig. 36.

It is likely that in crabs that utilize a proton gradient across the apical membrane of the epithelial cell to accomplish NaCl uptake from highly diluted media, such as the partially limnic Chinese crab *Eriocheir sinensis* or the true freshwater crab *Dilocarcinus pagei*, NH_3 diffuses across the apical membrane along its partial pressure gradient, as shown, for instance, in freshwater rainbow trout *Oncorhynchus mykiss* (2007). As mentioned above, mRNA expression of Rh-like ammonia transporters have been shown in gills and pleopods of many different crustaceans. Due to the lack of specific antibodies, the cellular localization of this putative ammonia transporter is unclear.

One can further speculate that in marine and brackish-water crabs, the Rh-proteins are not localized in the apical membrane of the gill epithelium, since here an apically H^+-ATPase, which generates an outwardly directed P_{NH3}, is missing. Ammonia (NH_3/NH_4^+) permeable structures would be a disadvantage, allowing ammonia influxes when the animals are exposed to high external ammonia concentrations. Many aquatic crab species are benthic living animals, hiding under stones or burying themselves in the sediment for long periods, for example during low tide or in the winter season. Under conditions where crabs are situated at sites with low rates of ambient water exchange plus the fact that the animals produce and excrete metabolic ammonia, concentrations of the ambient ammonia can reach high values.

Ammonia excretion in terrestrial crabs The mechanisms by which air-breathing crustaceans excrete nitrogenous waste into the terrestrial habitat have been investigated intensively (642, 643, 1348, 2017, 2020). Terrestrial crabs seems to tolerate considerably greater hemolymph ammonia loads when compared to aquatic species (1950), likely because diluting ammonia to non-toxic levels in the urine might require an unsustainable water loss in a land crab (2018). In all land crabs examined to date, the gills have become adapted for reabsorption of salt from the primary urine directed through the branchial chamber (1254, 2017, 2019), allowing diffusive NH_3 loss and NH_4^+ extrusion in exchange for required ions from the urine. For land-living

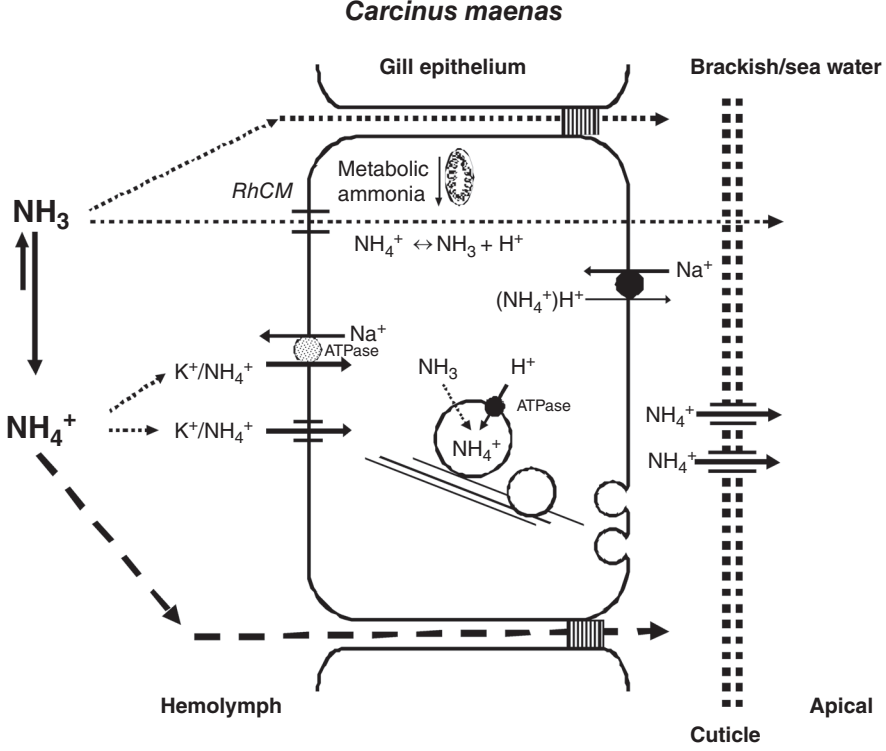

Figure 36 Branchial ammonia excretion model proposed for the green shore crab *Carcinus maenas*. The proposed mechanism is described in the text. The figure is modified after (1958).

crustaceans, different types of ammonia excretion modes have been described so far.

A "storage-excretion" mechanism was shown in diverse air-breathing crabs, for example in *Potamonautes warreni* (1255), in *Austrothelphusa transversa* (1073), in *Discoplax hirtipes* (416) and in *Cardisoma carnifex* (2022). *Discoplax hirtipes* excretes 99% of its waste as ammonia, but while breathing air, the rate of nitrogen loss in the urinary flow is only 0.2 μmol $kg^{-1}{\cdot}h^{-1}$ and NH_3 is volatilized at a very slow rate (0.4 μmol$\cdot kg^{-1}{\cdot}h^{-1}$). On reimmersion, the ammonia excretion rate is transiently elevated to 1,100 μmol$\cdot kg^{-1}{\cdot}h^{-1}$ compared to the normal aquatic excretion of 300 μmol$\cdot kg^{-1}{\cdot}h^{-1}$. *Potamonautes warreni* also does not excrete while in air, but on return to water the crab excretes ammonia across the gills at 4.9 mmol$\cdot kg^{-1}{\cdot}h^{-1}$ as compared to the normal rate in water of 70 μmol$\cdot kg^{-1}{\cdot}h^{-1}$. Gill irrigation appears to be a ubiquitous activity following excursions into the terrestrial environment (416). The requirement to reimmerse for branchial ammonia excretion may ultimately limit the duration of air breathing in amphibious species. However, *Austrothelphusa transversa* can spend many months without access to water without the need of ammonia excretion during that time period (1073). It was suggested that the near-cessation of nitrogen excretion in *A. transversa* implied reduced nitrogen catabolism and temporary nitrogen storage. The speed and brevity of the excretion pulse in *P. warreni* during immersion showed that wastes stored during terrestrial forays are rapidly excreted on return to water. Further investigations are required to determine the storage product, but an accessible intermediate such as glutamine is implicated. Apical H^+/NH_4^+ exchange and V-ATPase-driven cell alkalization have been suggested as likely mechanisms of transbranchial ammonia transport (1073).

Purine is stored in large amounts in connective tissue cells throughout the bodies of some land crabs (1071). In *Gecarcoidea natalis*, this stored purine is normally synthesized *de novo*, from excess dietary nitrogen. Recent data, including enzyme activities and nitrogen utilization (1072), have led to the suggestion of a storage-excretion function for the urate accumulated by *G. natalis* (644). Most land crabs recycle their urine over the branchial surfaces, producing a dilute fluid "P" (1253, 2019). The air-breathing ghost crab *Ocypode quadrata* also recycles the urine and produces "P", which can be as little as 10% of the osmotic strength of the primary urine. The ammonia concentration of the primary urine of the ghost crab is extraordinarily high. For example, in *O. quadrata* (419, 420), it reaches 116-212 mM and in *Ocypode ceratopthalma* and *Ocypode cordimanus* under field conditions, it reaches >40 mM and 27 mM, respectively. The primary urine of *O. quadrata* is rather acidic (pH = 5.36±0.21), providing an "acid trap" for NH_4^+. On passage over the gills, the pH is increased (pH = 7.01±0.24) and Cl^- (but not Na^+) is reclaimed, such that the alkalinization of the fluid promotes significant NH_3 volatilization (~71 μl$\cdot kg^{-1}{\cdot}h^{-1}$).

While pH 7 is not alkaline, the increase in pH is quite effective, promoting a nearly 40-fold increase in potential

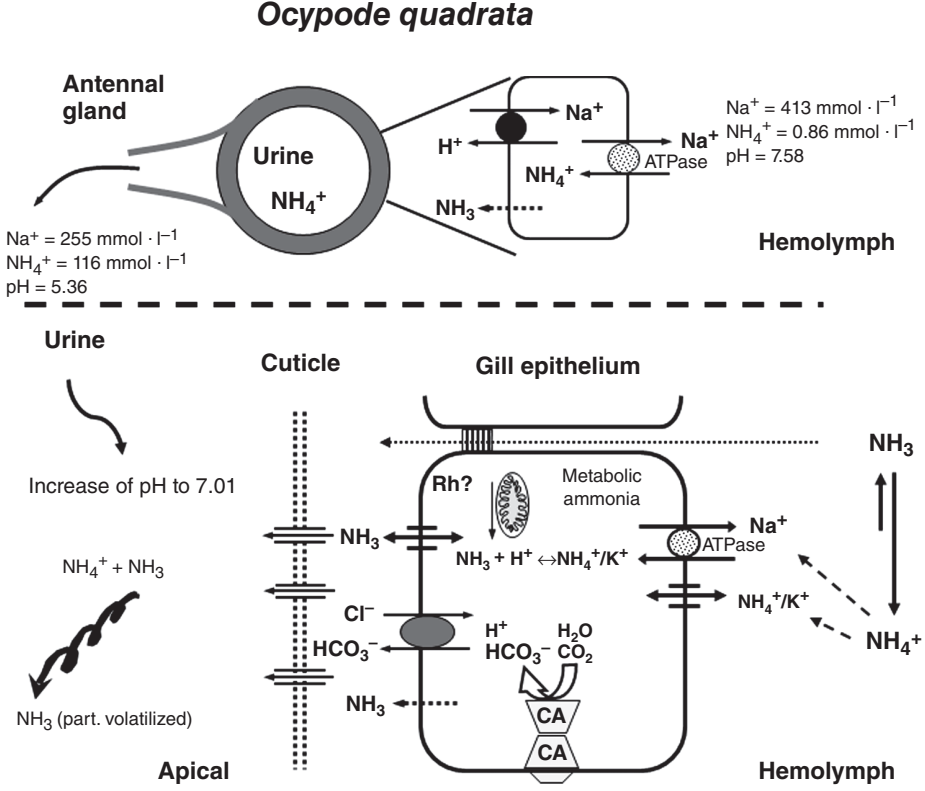

Figure 37 Ammonia excretion model in the terrestrial ghost crab *Ocypode quadrata*. The proposed mechanism is described in the text. The figure is modified after (1950).

diffusive gradient (P_{NH_3}). In view of the parallel increase in the fluid CO_2 concentration and the uptake of Cl^-, the most obvious candidate for the net base excretion is transport by an apical HCO_3^-/Cl^- exchanger (419). Reclamation of urinary Na^+ appears to be accomplished within the antennal gland (420). These authors report high activity of Na^+/K^+-ATPase in the antennal gland of *O. quadrata* for which NH_4^+ may substitute for K^+ in the basal membrane exchange. In addition, apical Na^+/H^+ antiporters in the antennal gland may sustain both Na^+ reclamation and acidification of urine to promote NH_4^+ trapping (Fig. 37).

Studies of the more terrestrial grapsid crab, *Geograpsus grayi,* have revealed modifications of the NH_3/NH_4^+ excretory system (646, 1904). *G. grayi* is a highly active carnivorous land crab and also reprocesses the urine to reclaim salts via branchial uptake (646), but unlike *Ocypode sp.*, it does not employ ion reclamation within the antennal gland (1904). *Geograpsus grayi* volatilizes NH_3 from the limited volume of "P" within the branchial chamber and thereby increases the effective NH_4^+ capacity of the fluid, which may achieve concentrations in excess of 80 mM compared with <1 mM in the urine (1904). However, *G. grayi* manages a rate of ammonia excretion comparable to that of aquatic crabs in water (107-220 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ [646, 1904]). Gaseous ammonia contributes ~78% of this total excretion in a discontinuous process over 3 hours to 3 days (1904), although urine flow is apparently limited, restricting fluid available for "P" formation. The pH of this fluid (pH = 8.07) is higher than that of the hemolymph (pH = 7.66-7.59), and at the same time the CO_2 content (36 $mmol \cdot l^{-1}$) is considerably greater than that of the hemolymph (13.7-17.2 $mmol \cdot l^{-1}$). Amiloride reduced NH_4^+ efflux by 83% in this system and reduced unidirectional Na^+ uptake. Thus, NH_3 volatilization is achieved by raising the fluid pH toward the pK such that gaseous NH_3 becomes 8% of the total ammonia, creating a diffusive gradient (P_{NH_3} ≈ 3.3 Pa) into the convective air stream (Fig. 38).

A net excretion reaction of

$$NH_4^+ + HCO_3^- \rightarrow H_2O + CO_2 \uparrow + NH_3 \uparrow$$

was proposed by Varley and Greenaway (1904). Since "acid ammonia trapping" does not occur in the gills of this crab, apical pathways for ammonia excretion might be similar to marine or brackish-water crabs, possibly via exocytosis. In fully terrestrial isopods, such as *Porcellio scaber*, NH_3 is volatilized from the surface of the abdomen, likely in a physiological coupling with active water vapor absorption (WVA) (2037). Ventilatory movements of the pleopods are observed during production of hyperosmotic fluid and WVA. Simultaneously with the production of WVA and pleopod movements, cyclic variations in total hemolymph ammonia were observed (2036). It seems that elevated hemolymph ammonia concentrations occur only under advantageous conditions, when atmospheric humidity allows WVA, and not humidity

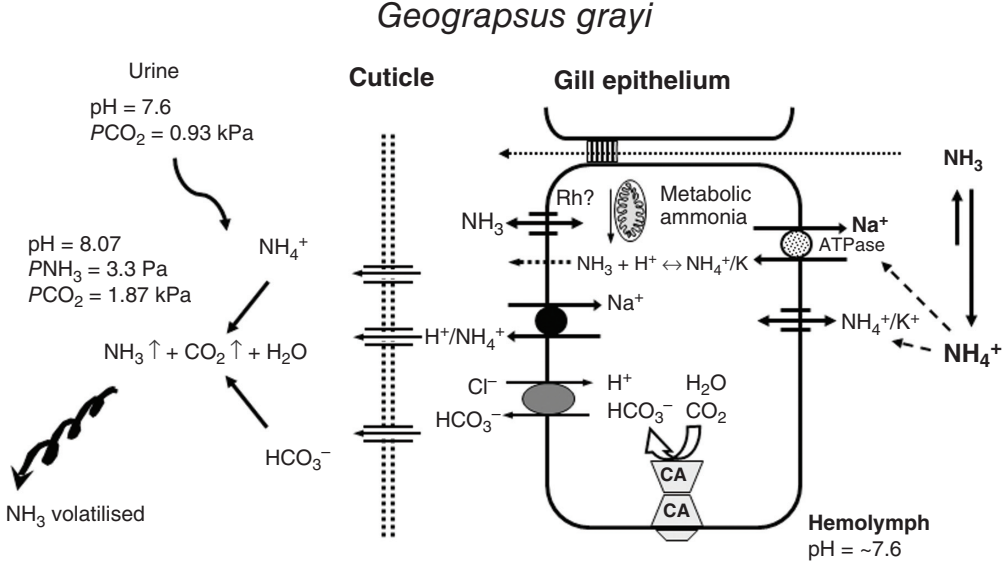

Figure 38 Ammonia excretion model in the terrestrial crab *Geograpsus grayi*. The proposed mechanism is described in the text. The figure is modified after (1950).

below of those appropriate for WVA. When conditions for ammonia excretion are disadvantageous, ammonia is stored as glutamine or arginine (2034, 2035). Transporters or mechanisms involved in hemolymph to surface ammonia excretion are unknown so far. The fact that expression of an Rh-protein was confirmed in the pleopods of the euryhaline isopod *Idotea baltica* (Weihrauch unpubl. GenBank accession#: AY094181) indicates that Rh-ammonia transporters most likely also play a role in ammonia excretion in terrestrial isopods.

The single known exception for a crustacean that is not ammoniotelic is the terrestrial anomuran robber crab *Birgus latro*, which is purinotelic and excreting 79.5% of its total excretory nitrogen as uric acid. The main route of excretion is via the feces, accounting for nearly 96% of the total N excretion (644, 645).

Urea excretion crustaceans With the exception of the uricotelic land-living robber crab *Birgus latro*, crustaceans are strictly ammoniotelic, and urea excretion usually accounts for no more than 20% of the total nitrogenous waste (417, 449, 844, 969, 1302, 1944). Concentrations of urea in the hemolymph are nonetheless relatively high and appear to be salinity dependent (1944). As shown for the green shore crab *Carcinus maenas,* hemolymph urea concentrations increase with decreasing salinity from ~20-80 µM in seawater to ~600-1,000 µM in crabs inhabiting brackish water (10 ppt salinity). In freshwater-living Chinese mitten crabs *Eriocheir sinensis*, hemolymph urea concentration of ~800 µM were detected (1749, 1944). Moreover, an increase of hemolymph urea in ammonia exposed juvenile *E. sinensis* suggests urea synthesis, and therefore ammonia detoxification, as an acute response to surging hemolymph ammonia during high exposure (795). As mentioned above, an EST project employing cDNA generated from gills of the blue crab *Callinectes sapidus* revealed the presence of a putative branchial urea transporter (Schaefer, EST project, GenBank accession #: CV527852). Gill perfusion experiments on closely related green shore crabs showed that urea is not excreted via the branchial epithelia (1951). The physiological relevance of this urea retention behavior in crabs acclimated to low salinities remains puzzling, since maximal hemolymph urea concentration of around 1 mM in brackish-water-acclimated *C. maenas* and freshwater-acclimated *E. sinensis* (1944) are likely too low to be used for osmoregulatory purposes.

Insecta

As reviewed by Pant (1398), insects are considered in general to be uricotelic, as an adaptation to their rather terrestrial lifestyle. In most terrestrial insects, 80% of the nitrogenous waste is excreted as uric acid. Accordingly, the amount of other nitrogenous waste products such as urea or ammonia was thought to be small. However, as pointed out by Harrison and Phillips (703), in earlier days the amount of excreted ammonia was likely strongly underestimated since the excretion products (pellets) were often oven-dried for analysis, which led to volatilization and loss of gaseous ammonia. Further, procedures for the analysis of ammonia from excretory pellets were not advanced enough to detect the molecule in its precipitated form.

There are now a number of reports showing that excretion of ammonia plays a considerable role as an excretion product in insects. Ammonotelism has been shown for aquatic larval stages of insects, for example for lacewings, *Sialis lutaria* (1750) and dragon fly nymphs of *Aeshna cyanea* (1751). The freshwater-inhabiting larvae of the yellow fever mosquito *Aedes agypti* also excrete ammonia. Employing

ion-selective electrodes, it was shown that ammonia excretion occurs in the osmoregulatory active anal papillae. It was suggested that ammonia excretion occurs by ammonia trapping, since similar to the findings in freshwater fish gills (see above), here also acidification of the unstirred boundary layer was observed (442), mediated likely by the activity of the apically localized H^+-ATPase (1407). It is conceivable that hemolymph ammonia is actively transported into the epithelia cell cytoplasm of the anal papillae via the basolaterally localized Na^+/K^+-ATPase (1407). It is noteworthy that two distinct Rh-like ammonia transporters were identified in *Aedes aegypti*, sharing 87% identity in amino acid sequence to each other and approximately 50% identity to trout Rhbg, in which ammonia transport capability was verified. Both of these putative ammonia transporters are expressed in the anal papillae, exhibiting a decrease in expression levels when larvae were exposed to elevated environmental ammonia concentrations (1948).

Excretion of ammonia by the larvae of terrestrial insects has also been described. Larvae of the screwworm fly, *Wohlfahrtia vigil*, infest open wounds in animal tissues and were found to be ammoniotelic after feeding on flesh (210). Ammonia excretion via the hindgut was shown for the larvae of the flesh fly *Sacrophaga bullata*, an insect that has to deal with high loads of ammonia due to the ingestion of rotting meat. The full mechanism of hindgut ammonia excretion in this species is unknown, but excretion appears to be active in the form of NH_4^+ (1490). Also for the tobacco hornworm *Manduca sexta* (Lepidoptera), ammoniotelism was suggested since high concentrations of ammonia (~60 mM) were found in the hindgut content (1943). In contrast to the findings in *Sacrophaga bullata,* the hindgut of *Manduca* larvae does not exhibit active ammonia excretion. However, mRNA expression studies revealed high abundance of an Rh-like ammonia transporter (RhMS). Since high mRNA expression levels of RhMS and H^+-ATPase were found also in the Mapighian tubules and tissues/lumen ammonia concentrations were high (~25 mM; Weihrauch, unpublished data), it was suggested that excess hemolymph ammonia is secreted into the Mapighian tubules and then finally released for excretion into the hindgut (144, 1943). The species of the nitrogenous waste in insect larvae, however, depends on the food source and availability of water. For instance, ryegrass fed cranefly larva *Tipula paludosa* are clearly uricotelic, excreting close to four times more uric acid-N than ammonia-N (650).

Adult terrestrial insects also excrete considerable amounts of ammonia as summarized in detail by O'Donnell (1338) and Weihrauch et al. (1948). For instance, in desert locusts, ammonia concentrations in the feces were exceptional high (up to 150 mM) (704), suggesting ammonotely. It was further shown that hindgut ammonia excretion was constant over a luminal pH-range of 7-5 and sensitive to amiloride, involving likely a Na^+/NH_4^+ transporter localized on the mucosal side (1824). Although the presence of an Rh-protein in the locust hindgut has not been shown yet, it is likely that here also this ammonia transporter is abundant and plays a role in ammonia excretion. Also cockroaches seem to be ammoniotelic. As shown for the American cockroach *Periplaneta americana*, up to 90% of their nitrogenous waste is excreted in the form of ammonia (1263). For blood-feeding insects, the type of the main excretory product might differ. While the tsetse fly *Glossina morsitans* predominately excretes uric acid and some amino acids such as arginine and histidine (228), adult yellow fever mosquitos *Aedes agypti* are ammoniotelic, excreting ammonia via the feces (1616). In order to detoxify high ammonia loads derived from amino acid metabolism after a blood meal, in addition to elevated ammonia excretion rates, glutamine and proline are synthesized as temporary non-toxic nitrogenous storage molecules. These amino acids are later utilized as important energy sources (622).

Chelicerata

The primary nitrogenous waste product in spiders, scorpions and uropygids is guanine (31, 799, 1512). However, an exception was found in *Paruroctonus mesaensis*. This desert scorpion excretes more than 90% of its nitrogenous waste the form of xanthine and is thereby xanthotelic (2058). The pathway for nitrogenous waste in arachnids appears to be via the Malpighian tubules/anal excretory system and not via the urine produced by the coxal gland, which is predominantly involved in ion and water balance (233, 234).

Urochordata

Many studies showed that ascidians, the most primitive living chordates, are predominately ammoniotelic (626), excreting up to 95% of their nitrogenous waste as ammonia. Beside this excretion product, ascidians retain also substantial amounts of uric acids and other purines, such as xanthine (627, 628, 1325). Some ascidians, but not *Ciona intestinales* or *Styela montereyensis*, excrete considerable amounts of urea as well (1158). At least in *Styela plicata*, urea excretion seems to be temperature dependent, increasing with declining temperatures, thus leading to a shift from ammoniotelism to ureotelism at temperatures below 20°C (536). In other ascidians as well, however—for example, in *Styela clava* and *Styela partita*—urea excretion is high, accounting for 47% and 42% of total excreted nitrogen at 20°C, respectively (1158). Ascidians are a particularly interesting species for analysis of nitrogen excretion since they bear gene information not only for mammalian-like ammonia transporter Rhag, Rhbg, Rhcg, but also for the rather primitive Rh-isoform RhP1 and at least two distinct AMT ammonia transporters (809). While the function in ammonia excretion of all these potential ammonia transporters needs to be evaluated, it was shown using gene knockout techniques that Ci-AMT1a is essential for brain development and brain function in *Ciona intestinalis* larvae (1157).

The subject of nitrogen excretion in fish has been reviewed and investigated extensively in recent years by a number of groups (72, 73, 191, 192, 813, 832, 1289, 1296-1300, 1608, 1873, 1927, 1928, 1953, 1996, 2041) and will only be summarized here.

Freshwater teleosts The predominant pathway for ammonia excretion in freshwater teleosts is by diffusion of ammonia gas down its partial pressure (P_{NH3}) gradient from blood to water across the gill epithelium. A large driving force in the current model of freshwater teleost ammonia excretion is the V-ATPase-energized acidification of the gill water boundary layer serving to readily protonate any NH_3 arriving at this layer to form NH_4^+, thus keeping the P_{NH3} in this boundary layer low (Fig. 34B). In addition, the hydration of CO_2, which crosses the gill membrane as CO_2 gas and forms H^+ and HCO_3^-, might also account for an apical acidification and ammonia trapping. For the rainbow trout *Oncorhynchus mykiss* Nawata et al. (814) identified seven full-length cDNAs, including one Rhag and two each of Rhbg, Rhcg and Rh30-like. Rhbg and Rhcg-1 and -2 were expressed in the gill. Functional expression analysis revealed that all of the trout Rh-proteins, except Rh30-like, facilitated ^{14}C-methylamine uptake when expressed in frog oocytes. Further, by employing NH_4^+-selective microelectrodes in conjunction with the scanning ion electrode technique (SIET), it was confirmed for Rhag and Rhcg2 that these proteins indeed mediate the transport of ammonia molecules. It was proposed that in trout, Rh-glycoproteins are low-affinity, high-capacity ammonia transporters (1300). Experiments exposing trout to elevated environmental ammonia concentration gave the initial clues of the relevance of the different Rh-proteins and other transporters in fish gill ammonia excretion. When fish were exposed to 1.5 mM NH_4HCO_3 in the surrounding water, an initial uptake of ammonia into the plasma was observed, resulting in increased plasma ammonia levels. When plasma levels reached a certain threshold (~500 μmol·l^{-1}) after approximately 12 hrs of exposure, ammonia net flux reverted, showing a net excretion. After 48 hrs of exposure, however, plasma ammonia levels (~1,000 μM) were only slightly below the concentration in the water, and excretion rates were highest. During this 48-hr period of ammonia exposure, mRNA expression patterns of ammonia transporting proteins were quite different in branchial mitochondria-rich cells (MRC) and pavement cells (PVC). While mRNA expression of Rhbg, Rhcg1 and Rhcg2 did not change in MRC, a significant increase of mRNA expression of Rhbg (about 4-fold after 48 hrs) and Rhcg2 (about 8-10-fold after 12 hr and 48 hr) was detected in PVC. Messenger RNA expression levels of Rhcg1 did not change in PVC, but increased for the H^+-ATPase (B-subunit) and Na^+/K^+-ATPase (α1a-subunit). In contrast, mRNA for Na^+/K^+-ATPase in MRC revealed a significantly lower expression level after 48 hrs of exposure. In both cell types, a decrease of mRNA coding for the cytoplasmic carbonic anhydrase-2 (CA2) was observed, concomitant with decreased CA2 activity rates in whole cell homogenates. This finding is puzzling since CA-catalyzed hydration of CO_2 normally fueled the H^+-ATPase in trout gill (1059), which is in turn important for branchial ammonia excretion (2007). It was suggested that under exposure to high environmental ammonia, the H^+ pump is fueled with protons generated not by CA activity, but by dissociation of NH_4^+ that might be transported at increased rates by the basolateral Na^+/K^+-ATPase from the plasma into the epithelial cells. Direct evidence for participation of the Na^+/K^+-ATPase in ammonia excretion in trout gill remains to be provided. However, it is strongly suggested that the pavement cells play the dominant role in branchial ammonia excretion. A hypothetical model of branchial ammonia excretion in pavement cells is presented in Fig. 39. The presence of apically localized Na^+/H^+ exchanger NHE2 and NHE3 in branchial Na^+/K^+-ATPase-positive MR cells in rainbow trout (835) led to the idea that in freshwater fish, ammonia excretion and Na^+ uptake function together in an apical Na^+/NH_4^+ exchange complex. It is thought that this complex, which consists of a H^+-ATPase, NHE2 and/or NHE3, Rhcg and Na^+ channel, works together as a "metabolon" that provides the acid-trapping mechanism for apical ammonia excretion. Indirectly this metabolon is supported by a carbonic anhydrase, providing H^+ ions and the basolateral Na^+/K^+-ATPase that accepts also NH_4^+ as a substrate (2041). Such a metabolon can also be assumed for the epidermal ammonia excretion mechanism in the planarian *S. mediterranea* (1947, 2038, Fig. 35), and represents possibly a general mechanism for ammonia excretion in freshwater animals.

Investigated by using a scanning ion-selective electrode (SIET), it was shown that in freshwater zebrafish larvae (five days post-fertilization), ammonia excretion occurs via the skin, in this case specifically via H^+ pump-rich cells and to a minor extent in keratinocytes and other types of ionocytes in larval skin. Highest ammonia excretion rates were detected in the yolk sac area. The NH_4^+ secretion was tightly linked to acid secretion and was reduced when the low apical pH generated by the proton pump was increased by a strong buffer in the media or by direct inhibition of the H^+-ATPase. A knockdown of Rhcg1 with morpholino-oligonucleotides also reduced NH_4^+ secretion significantly, without effecting H^+ secretion (1665). All these findings indicate that ammonia excretion in larval skin epithelia cells occur through an acid-trapping mechanism including the participation of Rhcg1. A parallel study on zebrafish larvae measuring whole animal ammonia excretion after knockdown of Rhag, Rhbg or Rhcg1 expression showed that silencing each of these Rh-proteins caused, independently from each other, a reduction of ~50% in ammonia excretion rates confirming importance of the Rh-proteins in the excretion mechanism in early life history stages as well (191).

In addition to ammonia, zebrafish larvae also excrete urea that at the life stages after hatching accounts for ~20-30%

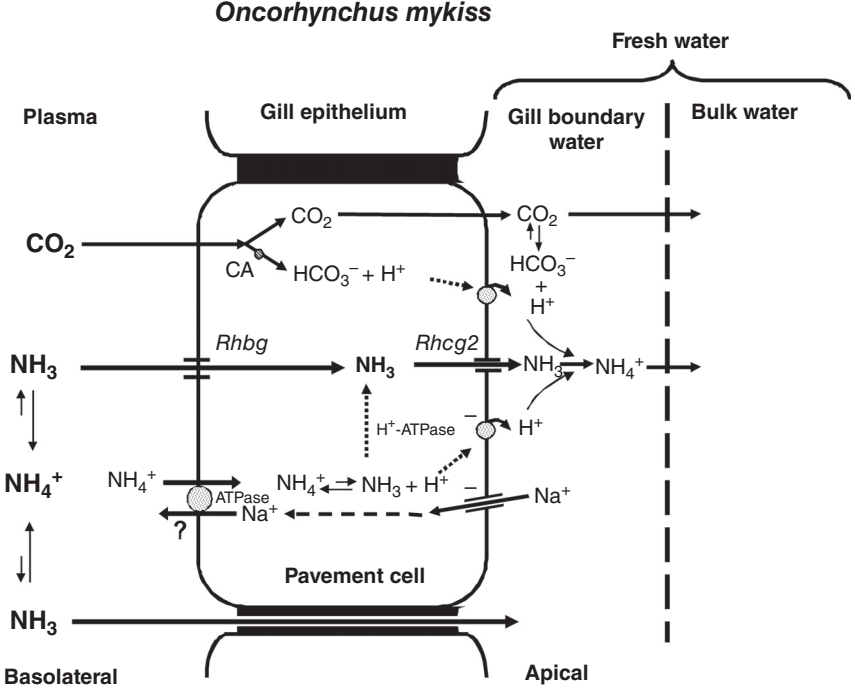

Figure 39 Branchial ammonia excretion model proposed for the rainbow trout *Oncorhynchus mykiss*. The proposed mechanism is described in the text. The figure is modified after (1953).

of total N excretion (191). This urea excretion was reduced by 90% relative to controls when the urea transporter (UT) was silenced, underpinning the importance of UT in nitrogen excretion. It seems that the synthesis of urea in freshwater fish larvae serves as an important backup detoxification system for ammonia. Indeed, when ammonia excretion is compromised and reduced, for example, due to the knockdown of Rhcg1, whole animal urea excretion rates increased nearly twofold (191).

Also, for at least two adult teleost fish species—the gulf toadfish (*Opsanus beta*) and the Lake Magadi Tilapia (*Alcolapia grahami*)—it has been shown that some or all of their nitrogenous waste is excreted as urea. The Lake Magadi Tilapia excretes nearly all of its nitrogenous waste as urea (1509) and exhibits a complete ornithine-urea cycle in several tissues (1068). In alkaline environments, the low levels of protons in the external water reduce the conversion of NH_3 to NH_4^+, thus minimizing the outwardly dircted P_{NH3} and thereby slowing the overall rate of NH_3 excretion.

Marine teleosts Branchial ammonia excretion in marine teleosts is considered to be quite different when compared to freshwater fish. Ammonia excretion by ammonia trapping due to the acidification of the unstirred gill boundary layer is likely compromised in seawater because of the increased buffering capacities of seawater. Also, since the gill of marine fish is considered to be more permeable to small ions and other molecules, paracellular pathways might play a role, in addition to the substitution of NH_4^+ ions for K^+ or H^+ on other ionic transporters. In the pufferfish *Takifugu rubripes*, a typical seawater fish, Nakada et al. identified piscine homologues to Rhag, Rhbg, Rhcg1 and Rhcg2 (1289). It was demonstrated that all isoforms are capable of the transport of the ammonia analog, methylammonia, when expressed in *Xenopus* oocytes. Interestingly, in *T. rubripes*, all Rh-proteins were expressed in the gill, with Rhag expression in the gill occurring in the pillar cells lining the vasculature and supporting the overlying pavement cells. Within the pavement cells Rhbg was expressed in the basolateral membranes and Rhcg2 was expressed in the apical membranes. Further, while Rhcg1 was expressed in the apical membrane of the chloride cells, no Rh proteins could be detected in the basolateral membranes of this cell type. Given the very large fractional surface area of the gill that is covered by pavement cells, one can predict that these cells will dominate in both mode and quantity of ammonia transported. Experiments exposing pufferfish *T. rubripes* to high environmental ammonia (1 mM NH_4HCO_3) provided further insights into the suite of transporters likely involved in branchial ammonia transport. Under these conditions plasma ammonia concentrations increased significantly from ~350 μM to ~800 μM. At the same time mRNA expression of branchial Rhbg was downregulated, while MRC Rhcg1 was strongly upregulated after 48 hrs of exposure. It should be mentioned that under these conditions ammonia was still excreted at rates similar to control rates. Along with changes in Rh gene expression, there was also an upregulation of the branchial H^+-ATPase, NHE3, NKCC and the Na^+/K^+-ATPase (1296). Both Na^+/K^+-ATPase and

NKCC have been shown to be expressed in MRC in seawater fish gill (885, 1201, 1417, 1905). No evidence for gill cell specific localization of H^+-ATPase and NHE3 has been presented to date for seawater fish; however, apical localization of these transporters in MRC of freshwater fish suggest a similar cell specificity in seawater fish, at least when the fish are ammonia stressed. According to Nawata and others (1296), ammonia stress activates ammonia excretion mechanisms in MCR, where ammonia is transported actively via the Na^+/K^+-ATPase from the plasma into the MRC cytoplasm. Direct participation of the Na^+/K^+-ATPase in ammonia transport was supported by enzyme assays that showed that this pump exhibited high activity when K^+ ions were replaced with NH_4^+. Plasma-to-cytoplasm transport of ammonia via basolateral NKCC might be driven by the Na^+/K^+-ATPase at the same time. The upregulation of H^+-ATPase and NHE3 (NHE2 is downregulated under ammonia stress) suggests an ammonia-trapping mechanism on the apical side by acidification of the unstirred gill boundary layer. This speculation is supported by the fact that in seawater fish, MRC is excreting ions into an apical crypt (514), a morphological structure composed of MRC and accessory cells, where water exchange by ventilation is likely limited and seawater pH buffer capacities are easily exhausted. Presence of an apical localized H^+-ATPase is at this point speculative and needs to be verified (Fig. 40). An apical localization of NHE3 in MRC of the amphibious mudskipper *Periophthalmodon schlosseri* is known (508), and at least under environmental ammonia stress conditions, it was suggested for this fish that active ammonia is mediated via an apical NHE and Na^+/K^+-ATPase (1508). Also in this species an apically localized H^+-ATPase was detected in MRC (2006). Further, the evidence of expression of Rhbg and Rhcg2 in the skin of *T. rubripes* suggests that, in addition to the gills, the skin also plays a certain role in ammonia excretion in marine fish (1296).

In addition to ammonia, substantial excretion of urea has been found in the gulf toadfish *Opsanus beta,* a saltwater teleost. *Opsanus beta* can excrete urea in distinct pulses mainly through a UT-mediated pathway in the gills (1204, 2021), and in addition to Rhbg, Rhcg expression of an functional urea transporter (tUT) has been verified in the gills of the fish (1885) (1906). In their natural habitat gulf toadfish excrete roughly similar amounts of ammonia and urea (72, 73). Some evidence suggests that urea excretion functions to hide the scent of ammonia to predators by the cloaking molecule urea (73).

Ammonia excretion in amphibious teleosts In air, NH_3 cannot be easily hydrated, which would negate ammonia trapping across epithelial tissues such as the skin or gills. Amphibious fishes deal with these problems by either tolerating extremely high internal concentrations of ammonia, found in fishes such as the oriental weatherloach (*Misgurnus anguillicaudatus*; [1874]), or by detoxifying ammonia to less toxic waste end-products such as glutamine or urea as is seen in the lungfishes (282, 842, 843, 1715). There are a number of fishes able to excrete ammonia in air. The giant mudskipper (*Periopthalmodon schlosseri*) and the climbing perch (*Anabas testudineus*) appear to use active NH_4^+ excretion to excrete

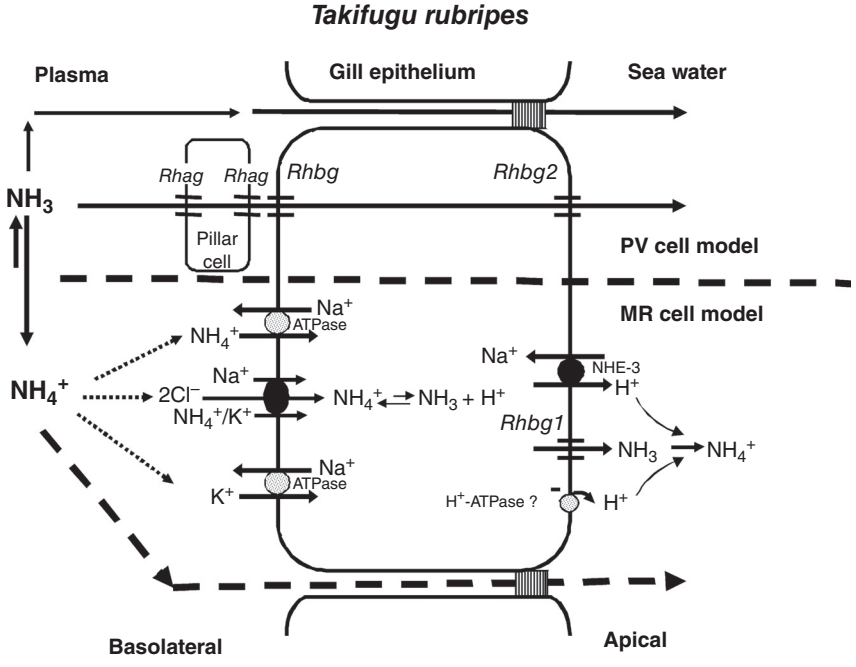

Figure 40 Ammonia excretion model proposed for pavement and mitochondria-rich cells in the puffer fish *Takifugu rubripes*. The proposed mechanism is described in the text. The figure is modified after (1953) and (1289).

ammonia against massive inwardly directed P_{NH3} and NH_4^+ electrochemical gradients (283, 1508, 1809).

The giant mudskipper is an obligatory air-breathing fish that drowns if denied access to air (1508). Experiments employing ouabain suggested basolateral NH_4^+ transport takes place via the Na^+/K^+-ATPase (Randall, 1994), which possibly also powers basolateral NH_4^+ uptake by a NKCC cotransporter (950, 1506). Both transporters have been localized to the basolateral membrane of MRC via immunohistochemical studies (2006). Ammonia excretion is also amiloride sensitive, suggesting an apical Na^+/H^+ (NH_4^+) exchange via NHE. The retention of saltwater within the confines of chambers formed by the fused lamellae of in the gill of the giant mudskipper possibly provides the needed inwardly directed Na^+ electrochemical gradient to energize the process (2003). Localization of an H^+ATPase to the apical crypt region of MRC suggests that this pump also participates in ammonia excretion, probably by ammonia trapping. However, buffering the apical solution had no effect on the excretion rates (2006). In contrast, partial reduction of the ammonia excretion rate was accomplished employing acetazolamide, indicating the participation of the carbonic anhydrase in this process.

Although not demonstrated yet, it is likely that in the MRC of the mudskipper, as shown for MRC in other teleost fish, an apically Rhcg1 is present and plays a role in ammonia excretion. Experiments by Chew and others showed that only a small percentage of plasma ammonia is detoxified to urea during emersion (283) (Fig. 41). Ammonia volatilization across the skin of amphibious fish has been reported for the air-breathing tropical fishes, the Mangrove killifish *Kryptolebias marmoratus* (557, 558) and the oriental weatherloach *Misgurnus anguillicaudatus* (1874). The weatherloach prefers the muddy or sandy bottoms of lakes, ponds and rice fields, and will migrate overland when necessary if water is scarce (833). Tsui et al. (1874) showed that the skin of *M. anguillicaudatus* becomes alkalinized by approximately 1.6 pH units, leading to the suggestion that this fish volatilizes ammonia as NH_3 during air exposure. Although traces of volatilized NH_3 were measured, the overall importance of cutaneous ammonia excretion in this animal is not clear. To achieve ammonia volatilization, plasma ammonia levels would have to reach very high concentrations to generate the needed P_{NH3} gradients. In fact, the oriental weatherloach is able to tolerate plasma total ammonia concentrations of ca. 5 $mmol \cdot l^{-1}$, making it one of the most ammonia-tolerant fishes known so far (281, 1874).

The mangrove killifish *K. marmoratusi* inhabits intertidal zones and mangrove swamps, and may occasionally be immersed as it hides among leaves and other debris in this habitat (557). *K. marmoratusi* volatilizes NH_3 across its body surface when exposed to air under humid conditions, likely to prevent or minimize the accumulation of ammonia within the tissues (557, 1081). It was shown by (558) that approximately 40-50% of the ammonia excreted by this animal during air exposure was across the back end of the body. During air exposure the cutaneous surface is alkalinized by 0.4 to 0.5 units (1081), thus promoting the accumulation of NH_3 on the skin surface. It was proposed that slight air currents would be sufficient to reduce the boundary layers and volatize the NH_3 at the skin surface, where a P_{NH3} of approximating 1200 μTorr was measured.

The mode of ammonia excretion across the skin of the mudskipper is still puzzling. Hung et al. (814) demonstrated mRNA presence of Rhbg, Rhcg1 and Rhcg2 in the skin of the fish, of which Rhcg1 and Rhcg2 were upregulated several-fold when fish were exposed to air (24 hrs). Assuming that Rhcg1

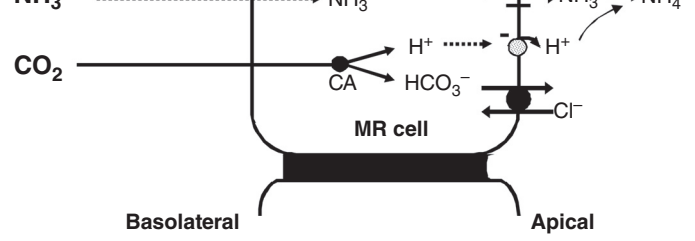

Figure 41 Branchial ammonia excretion model proposed for mitochondria-rich cells in the giant mudskipper *Periophthalmodon schlosseri*. The proposed mechanism is described in the text. The figure is modified after (1953).

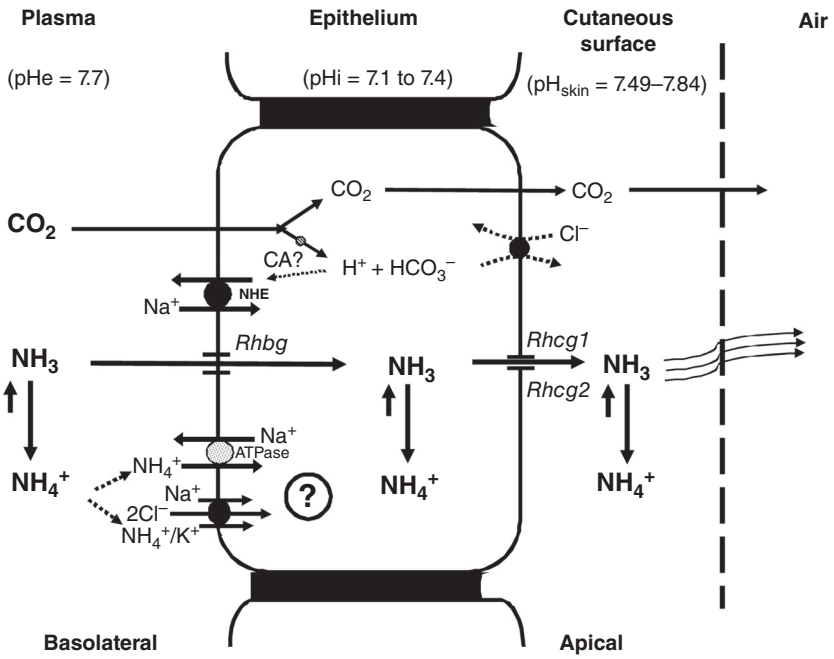

Figure 42 Proposed ammonia excretion across the skin of the Mangrove killifish *Kryptolebias marmoratus*. The proposed mechanism is described in the text. The figure is modified after (1953).

and Rhcg2 promote NH_3 transport, apical alkalinization of the skin could be accomplished either by apical NH_3 excretion or HCO_3^- secretion (the latter mediated by the action of an intracellular carbonic anhydrase) or both mechanisms. It is likely that for this mechanism to function, intracellular H^+ generated by CA must be eliminated back into the plasma possibly via a basolateral NHE. It was speculated that basolateral plasma to cytoplasm ammonia uptake occurs actively via the Na^+/K^+-ATPase and maybe NK(NH_4^+)CC cotransport, which could provide an apical outwardly directed ammonia gradient (1081). Basolateral participation of a Na^+/K^+-ATPase in ammonia excretion is indeed conceivable since most animal epithelia cells express this pump, which is apparently involved in most active ammonia transport mechanisms, as reviewed in this article. A hypothetical model of cutanous ammonia excretion in the mangrove killifish is illustrated in Fig. 42.

Elasmobranchs As elaborated in a separate section of this article, marine elasmobranch fishes, such as sharks, rays and skates, retain more than 300 mM urea in the extracellular fluids, which is used as osmotic ballast (1716). As a consequence, a large concentration gradient for urea exists between the plasma fluids and the environment, resulting in a passive loss of urea (164). It is because of this leakage that marine elasmobranchs are ureotelic. For instance, in the spiny dogfish *Squalus acanthias*, more than 90% of the nitrogenous waste is excreted in the form of urea and less than 5% as ammonia (2021). The main site of urea excretion is the gills, accounting for nearly 95% of the total urea loss from the fish (2021). Although urea transporters have been identified on a molecular basis in elasmobranchs (34), expression in the gills has not been demonstrated. In contrast, experiments on basolateral membrane vesicles from dogfish gills suggest the presence a phloretin-sensitive sodium-coupled urea transporter, thought to be involved in the reclamation of urea that has leaked into the epithelia cells (531).

The role of ammonia excretion in elasmobranchs is not well understood yet. For pups of the spiny dogfish it was calculated that about 77% of total branchial ammonia efflux was via non-ionic diffusion of NH_3 and ~6% as diffusion of NH_4^+, likely paracelluar (511). Employing bumetanide, a small but significant inhibition of ammonia excretion was detected, suggesting the participation of a basolateral NKCC. In the gills of the chondrichthyan ratfish *Hydrolagus colliei*, molecular evidence for an Rh-like ammonia transporter was found (36). Whether this branchial ammonia transporter is used for ammonia excretion or ammonia retention remains to be investigated.

Amphibia The subject of nitrogen excretion in amphibians has been studied by many investigators, with the most recent review by Wells (1964). Amphibians exhibit an exceptional plasticity in their nitrogen excretion strategies. For instance, the Bornean toad *Bufo quadriporcatus* dwells mostly on the forest floor and is ureotelic when on land, but switches to ammonotely when placed in water (1674, 1964). Other

amphibians, for example the toad *Bufo asper* or the frog *Limnonectes blythii*, show a lesser degree of flexibility with regard to the form of nitrogen excreted. Both species are usually found along river banks; however, they also remain ureotelic when placed in water (1674). Embryos and larvae of anurans and urodeles are usually aquatic and commonly excrete ammonia as the primary nitrogenous waste product. However, larvae of species that develop in dry conditions or in surroundings with only limited water exchange are found to be ureotelic. For instance, larvae of the Victorian smooth froglet *Geocrina victoriana*, a species with terrestrial eggs, excrete up to 86% of their nitrogenous waste as urea (1171). Also, embryos of amphibians developing in pouches on the back of a marsupial frog such as *Gastrotheca riobambae* (17) or in the uterine fluid inside of the reproductive tract of the ovoviviparous *Salamandra salamandra* (1621) are ureotelic. Some frogs, for example the Argentinian frog *Leptodactylus bufonius* (1675) or the African pig-nosed frog *Hemisus marmoratus*, lay their eggs in foam nests, where their larvae develop. The tadpoles of *H. marmoratus* excrete about 80% of the nitrogenous waste in form of urea (637). Clearly exceptional for amphibians is the excretion of uric acid as the main nitrogenous waste product. Uricotely has been decribed so far only in "waterproof frogs" in the genera Chiromantis (e.g., the South African Grey foam-nested tree frog *Chiromantis xerampelina*) and Phyllomendusa (1100, 1668, 1672). The arboreal Waxy Monkey Leaf Frog, *Phyllomedusa sauvagii*, lays its eggs not into water bodies but on leaves. During early stages of development, tadpoles of *P. sauvagii* excrete similar amounts of ammonia and urea, but gradually switch toward ureotely in later stages. While ammonia excretion rates further decrease over time, uric acid release is initiated and can be detected even before completion of metamorphosis (1673, 1964). Semiaquatic and terrestrial amphibians are generally considered to be ureotelic when reaching adulthood. However, along with urea, some ammonia is excreted (351, 2031). Fully aquatic frogs, such as *Xenopus laevis*, or amphibians that do not complete metamorphosis from fully aquatic larvae stages to the "adult" semiterrestrial life form, such as the mudpuppy *Necturus maculosus*, remain ammoniotelic (351, 516, 2031).

In typical frogs such as *Rana*, ammonia excretion occurs in the kidney by an active secretion in the distal tubules and collecting ducts (1920). However, in fully aquatic and semiaquatic amphibians, ammonia is also partially excreted across the skin (68, 551, 582, 1766, 1903). In the mudpuppy *Necturus maculosus*, up to 90% of body fluid ammonia is excreted via the skin, and 10% ammonia plus urea is eliminated by the kidney (516). The aquatic caecilian "rubber eel" *Typhonectes natans* excrete similar amounts of their nitrogenous waste in form of ammonia and urea, with both molecules (~90% of ammonia and ~70% of urea) being predominately eliminated via the skin (1766). Also for semiaquatic frogs such as *Leptodactylus ocellatus* (1573), *Rana esculenta* (1493) and the Northern Leopard frog *Rana pipiens*, ammonia excretion via the skin was documented. Skin excretion rates in *R. pipiens* could be stimulated by applying an internal ammonia load or by plasma obtained from a dog during metabolic acidosis (551). The precise mechanism responsible for ammonia excretion via the amphibian skin is unknown at this moment. However, it is conceivable that ammonia is eliminated by ammonia trapping in a similar fashion as described in freshwater fish gills (see above). The model illustrating the hypothesis for osmoregulatory NaCl uptake across frog skin (see section on Amphibia, Fig. 21) depicts two cell types: MR cells containing an apical H^+-ATPase and basolateral Na^+/K^+-ATPase, and electrically coupled principal (granular) cells containing a basolateral Na^+/K^+-ATPase. Moreover, genome projects and ESTs on *Xenopus tropicalis* and *Xenopus laevis* revealed the presence of genes coding for the ammonia transporters Rhag, Rhbg, Rhcg and RhP1 (809). Indeed, a recent study revealed that cutaneous ammonia excretion in *X. laevis* (~50% of total excretion) is active and can be enhanced by the phosphodiesterase blocker theophylline. Gene expression analysis and enzyme activity assays strongly suggest the participation of the Na^+/K^+-ATPase, H^+-ATPase, Rhcg and Rhbg in the cutaneous excretion process (362).

Experiments in the neotenic axolotl *Siredon mexicanum* suggest further that the gills, especially in smaller animals with well-developed vascular gills, are able to excrete waste nitrogen (1264).

Conclusions

The energetics of ion and water exchanges in aquatic animals constitutes one of the main themes of the article. Several types of ion motive ATPases are expressed in osmoregulatory organs of invertebrates and vertebrates. Among these ATPases, the distribution and function of the Na^+/K^+-ATPase discovered in the late 1950s have been studied in all major organs specialized for whole body osmoregulation such as kidneys, gills, antennal glands, integument, intestine and exocrine glands. In all animals studied, the "sodium pump" energizes the high extracellular Na^+/K^+ and low intracellular Na^+/K^+ concentration ratios. Cell water volume is determined to a large extent by the intracellular Cl^- pool maintained above thermodynamic equilibrium by the concerted and regulated activity of several mechanisms such as the Na^+/K^+ pump, the Na^+-$2Cl^-$-K^+ cotransporter and the passive flows of Cl^- and K^+ in specific channels in the plasma membrane. Studies within the last decades have indicated that the V-type H^+ pump, which plays a central role in energizing insect epithelial ion transport, plays specific roles in extracellular osmoregulation and acid-base balance of animals generally. In all epithelia investigated, the Na^+/K^+ pump is expressed at the membranes lining the lateral intercellular spaces that would allow for solute-coupled water transport, isotonic transport and uphill water transport. These functions have been studied in proximal nephron of vertebrate kidney, small intestine and anuran skin, which have been shown to transport water at transepithelial osmotic equilibrium. Experimental studies and quantitative biophysical analyses

of cellular energy expenditure in freshwater invertebrates and amphibians indicate that the concerted activity of the two above-mentioned ion motive ATPases would allow for uptake of NaCl from concentrations as low as 10^{-5} mol·l^{-1}. In some epithelia, additional ion motive ATPases fuel specific epithelial functions, as, for example, the Na^+/NH_4^+ ATPase in crustacean gills.

The comparative approach applied throughout this article permits the identification of common principles and considerations of the diversity of the cellular organization of osmoregulatory and excretory epithelia. This identification of common principles within diverse organization constitutes a second main theme of the article. The polarized epithelia constituting the interface between the extracellular fluid and the environment are multifunctional by serving extracellular ion- and water homeostasis, acid-base balance, and excretion of nitrogenous waste products. The associated thermodynamic work is fueled by hydrolysis of ATP at Na^+/K^+ pumps and H^+ pumps. Different aquaporin water channels are expressed at the luminal and basolateral membranes, and the regulation of transepithelial water transport takes place by trafficking of aquaporins between cytoplasmic pools and the apical membrane. Across taxonomic groups, osmoregulatory epithelia are multicellular with minority cell types, denoted intercalated- or mitochondria-rich (mitochondrion-rich) (MR) cells, interspersed between principal cells. Several types of MR cells have evolved not only across different phyla but also within a given taxonomic group as exemplified in the gills of freshwater teleosts. Special attention is paid to hypoosmoregulators in marine environments that depend on salt-secreting exocrine glands (reptiles, birds), gills (crustacea and teleost fish) and newly recognized specialized intestinal epithelia (teleost fish). Beyond the well-described segmental functional organization of the vertebrate nephron there are other examples of tightly coupled coordinated ion and water transport across epithelia associated anatomically, such as the complex of Malpighian tubule, midgut and rectum of insects, and probably also the epidermal epithelium and the subepidermal exocrine glands in the skin of frogs on land.

The third major theme of the article concerns whole body ion and water homeostasis resulting from balanced uptake and elimination of water and solutes governed by different osmoregulatory organs. The discussion aims at the diversity of adaptations and the mechanisms of acclimatization in species living in and migrating between environments of different osmotic conditions. The conclusions are summarized for individual taxonomic groups as outlined below.

Annelida and Mollusca

Most molluscs that live in seawater are osmotic conformers; these animals conserve energy at the organismal level by allowing the ionic concentration of the extracellular fluid to vary with the ambient concentration. The cost of this strategy is that each cell in the animal must retain the mechanisms and capacity to either increase or decrease the cytoplasmic osmotic concentration. Cellular regulation involves changes in cytoplasmic concentrations of both inorganic ions and organic molecules, such as amino acids. Species that live in brackish water often are osmotic conformers at higher salinities and hyperosmotic regulators at ambient salinities below 4-5. At lower salinities, the osmotic and ionic gradients between ambient and the body fluids are low compared to that in arthropods and the energetic cost of ion uptake and production of the hypo-osmotic cytoplasm is reduced. Terrestrial molluscs and annelids are subject to desiccation, and can survive the loss of considerable amounts of body water by evaporation. Withdrawal into the shell (if present) reduces water loss. These animals can take up water by osmosis across the body wall. Urine is produced by filtration. In annelids, nephridia are present and the number in each animal varies greatly among species across the phylum. In molluscs, the heart is the site of filtration and the filtrate is processed by a kidney.

Crustacea

The vast majority of crustaceans are aquatic animals of marine origin. Most marine crustaceans are osmoconformers, maintaining only minor osmotic gradients across their body surface. However, a number of these osmoconformers is considerably euryhaline, having the ability to migrate to more dilute ambient media on the basis of their capability for intracellular osmoregulation, adjusting the intracellular osmotic concentration to the values of the hemolymph and the ambient medium. Hyperosmoregulating crustacians evolved mechanisms for extracellular osmoregulation, allowing them to inhabit more dilute ambient media like freshwater and more diluted brackish waters. Apart of preventive mechanisms like reduced body surface permeabilities and the tolerance of reduced body fluid osmolalities, hyperosmoregulating crustaceans evolved transport mechanisms in branchial epithelia that compensate for the passive loss of salt to the dilute ambient media. Two different mechanisms of active NaCl absorption have been characterized. Like amphibians and fish, freshwater crustaceans appear to use epithelial Na^+ channels and V-type H^+-ATPases in the apical membrane of the gill epithelium. On the other hand, a mechanism involving apical Na^+-$2Cl^-$-K^+ cotransporters and K^+ channels and/or electroneutral cation and anion exchangers appears to dominate active NaCl absorption in hyperosmoregulating crustaceans in moderately dilute ambient media. The passive water gain in dilute waters is balanced by urine production. Whereas the latter considerably contributes to the overall salt loss in many hyperregulating crustaceans without capability to produce dilute urine, some crustaceans evolved transport mechanisms in the antennal glands to reduce this loss of salt by active absorption. Some crustaceans are hypoosmoregulators in the sea or in hypersaline media. It appears that the gills are the site of active salt secretion. However, the mechanisms are unknown and require further study. Whereas the gills are the major site of osmotic and ionic regulation in Crustacea,

the antennal glands, the gut and the hepatopancreas contribute to osmotic, ionic and pH homeostasis.

Insecta

The increased availability of oxygen and novel food sources in the form of flowering plants were important factors contributing to the explosive population expansion and speciation of terrestrial insects. Terrestriality also created the potential for desiccation, however, particularly for small animals with a high ratio of surface area to volume. There was thus a strong selection pressure for the evolution of extraordinarily effective mechanisms to reduce water loss from the gut, from the respiratory system and across the cuticle of the exoskeleton. Lipids in the epicuticular (outer) layer of the cuticle are the primary barrier to transpiratory water loss, analogous to the role of epidermal lipids in limiting water loss by reptiles (see *Reptiles*, below) and some terrestrial amphibians (see *Amphibians*, below). Along with the evolution of flight, these mechanisms for restricting water loss were major factors contributing to the success of insects on land. In contrast to the dominant role of the Na^+/K^+-ATPase in powering ion transport by vertebrate osmoregulatory epithelia, the vacuolar-type H^+-ATPase plays a preeminent role in driving secretion or absorption of ions across the plasma membranes of the gut and Malpighian (renal) tubules of insects. It is also worth noting that although plants provided potential food sources for insects, flowering plants evolved numerous toxins designed to minimize herbivory. In turn, powerful mechanisms for detoxification and excretion of such compounds have evolved in the insects. Microarray studies show that the genes for organic solute transporters dominate the array listing for the Malpighian tubules, consistent with a pivotal role for the tubules in elimination of toxins.

Agnata and Pisces

While freshwater fish are forced to regulate internal salt concentrations above ambient, all osmoregulatory strategies are represented among marine fishes. A high number of teleost species are euryhaline and osmoregulate efficiently in salinities ranging from freshwater to seawater, offering convenient models for the study of osmoregulatory physiology. Among these species killifish, several tilapia and salmonids have become model organisms. Much of what is known about osmoregulation by teleosts stems from this limited number of species as well as zebrafish, commonly used for studies of freshwater osmoregulation. Even within this limited number of species, it is already apparent that distinct mechanisms for ion uptake in hypoosmotic environments exist. Undoubtedly this diversity reflects a high number of freshwater invasions, and it follows that exploration of additional species in comparative approaches will yield additional insight into mechanisms responsible for freshwater osmoregulation in the most radiant group of vertebrates. The past few years have revealed novel insight into the mode by which ammonia excretion and Na^+ uptake is linked in freshwater fish. It is becoming clear, however, that this aspect of brachial transport in freshwater fish varies among species as well. So far it appears that mechanisms of salt and water transport are more consistent among marine species. However, this may simply reflect that significantly less attention has been devoted to marine species. Recent years have witnessed a departure from the tradition of doing studies of fish osmoregulation on unfed animals and have already revealed important roles of dietary salt intake in freshwater species as well as complicated interactions between processing of ingested food and osmoregulation in marine species. Epithelial barrier function afforded by claudins and occludin is arguably one of the most exciting novel areas of fish osmoregulation and is bound to receive considerable attention over the next decades.

Amphibia

Modern amphibians are adapted to almost all climatic zones and are found in the range from purely aquatic to purely terrestrial habitats. Generally, the body fluids are diluted as compared to other vertebrates, with ion concentrations submitted to larger variations. In freshwater, a high rate of glomerular filtration secures the cutaneous uptake of water being balanced by voiding of diluted urine. The water permeability of the urinary bladder is small, and reabsorption of water from the bladder urine is of marginal importance for body water balance. In dry environments, urine production decreases because of decreased glomerular filtration rate and increased tubular fractional water reabsorption. Urine is stored in the bladder and recycled into the body fluids governed by evaporation from the body surface. In anurans, water is taken up by cutaneous drinking, which at all levels, from behavior to the anatomical and functional organization of the skin, is an advanced specialization of terrestrial living. The regulation of water and ion exchange at the cell level of skin, kidney and urinary bladder has been studied extensively, leaving unanswered questions about how their functions are coordinated at body level. Adaptations at organ and cell levels have evolved specifically for living alternately in terrestrial and aquatic habitats. In freshwater, uptake of Na^+ by the principal cells and of Cl^- by MR cells is both active and fueled by the P-type Na^+/K^+-ATPase and the V-type H^+-ATPase, respectively. The frog on land maintains a slightly hypotonic cutaneous surface fluid by regulated subepidermal gland secretion, which is balanced by water evaporation into the atmosphere and ion and water reabsorption by principal and MR cells of the epidermis. These adaptations evolved *pari passu* with dynamic permeabilities of the skin controlled by external ion and osmotic concentrations. This allowed for fast switching of the cutaneous uptake of chloride between active and passive transport associated with a dynamic electrical coupling of active sodium uptake by principal cells and passive chloride uptake by MR cells. It is not known whether similar adaptations are present in all anurans and in salamanders and caecilians.

Reptilia

Although the solute and water composition of the *milieu intérieur* is not as tightly regulated in reptiles as in mammals and birds, it is still maintained within certain limits. The skin, kidneys, bladder, cloaca, colon and extrarenal salt glands all play important roles in this process. The function of these organs and their regulation with changes in the external environment differ among orders and species from different habitats. Much is known about the function of these organs, but there are still major gaps in our knowledge. Moreover, although significant information is available about the regulation of many specific routes of solute and water movement, the information is far from complete, and almost nothing is known about the comprehensive integrated regulation of all these routes together. Such integrated regulation is essential to the overall maintenance of the *milieu intérieur*.

Aves

Birds have sufficient osmoregulatory ability to inhabit all environments on earth while maintaining their water and electrolyte *milieu intérieur* within very narrow limits. Osmoregulation involves both renal and extrarenal mechanisms. Renal mechanisms involve changes in GFR, including changes in the number of filtering nephrons; changes in tubular reabsorption of salt and water, including production of a moderately concentrated urine; and excretion of uric acid as the primary end product of nitrogen metabolism. Extrarenal mechanisms involve modification of ureteral urine in the lower gastrointestinal tract and secretion of a concentrated sodium chloride solution by nasal salt glands. Although urine concentrating ability is limited, excretion of uric acid and the solute-coupled water absorption in the lower intestine make this limited concentrating ability highly appropriate to the conservation of water, and the nasal salt glands permit the additional excretion of sodium chloride and production of solute-free water in those marine species that require it. However, much still remains to be learned about the integration of these osmoregulatory mechanisms in the intact animal.

Excretion

Ammonia is a highly toxic waste product derived predominantly from protein metabolism. Due to its toxicity, ammonia needs to be either detoxified by ATP-demanding processes into less toxic molecules (e.g., urea or urate) or rapidly excreted to keep cellular and body fluid levels within a tolerable range. Which type of nitrogenous waste is excreted depends in general on the availability of water. While it was traditionally generalized that aquatic invertebrates and teleost fish are ammoniotelic, amphibians, elasmobranchs and mammals are ureotelic, and terrestrial invertebrates, birds and reptiles are uricotelic, there are a great number of exceptions to the rule, which can be found, for example, in insects and amphibians. This section reveals that our knowledge on nitrogen excretion and its underlying mechanisms in aquatic and terrestrial animals is by far not complete. Among other things, future studies will show whether or not H^+-ATPase and Rh-protein-mediated ammonia trapping is a general excretion strategy in freshwater animals and further reveal the relevance of a vesicular ammonia transport mechanism in animals inhabiting environments with a higher buffer capacity. Open questions also remain regarding the role and function of AMTs and aquaporins in nitrogen transporting epithelia.

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