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The Pump and Leak Steady-State Concept with a Variety of Regulated Leak Pathways

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Abstract. The paper will reflect on how Ussing has affected my own scientific work and how he created much of the framework within which I have been working. I have used five examples: (i) The first description of a 1:1 *exchange diffusion* was introduced by Ussing in 1947 and has been found to be of great physiological significance in most cells. We found that Cl^- transport in Ehrlich ascites tumor cells (EATC) was completely dominated by an exchange diffusion process, as defined by Ussing, and, thus, the Cl^- conductance was much lower than previously estimated from measurements of unidirectional tracer fluxes. This had a major influence on my later description of a swelling-activated Cl^- conductance. (ii) *The pump-leak steady-state concept* for cell volume control was introduced by Krogh in 1946, but it was developed in detail by Leaf and Ussing in 1959. This concept was the basis for me and others, when we later found that the passive ion leaks play an active role in cell volume control. (iii) The use of isotopes and Ussing's famous *flux ratio equation* provided an ingenious instrument for distinguishing the various transport routes. We used this to identify the Na,K,2Cl cotransport system as accounting for maintaining a $[Cl^-]_i$ in the EATC far above thermodynamic equilibrium, as well as accounting for the ion uptake during a regulatory volume increase (RVI) in EATC, similar to what Ussing had found in frog skin. (iv) *Short-circuit current setup in the Ussing chamber* is still used in laboratories around the world to study ion transport across epithelia. A few results on Cl^- transport across the operculum epithelium of the small eurohaline fish *Fundulus heteroclitus* mounted in small Ussing chambers are presented. (v) *Shrinkage-activated Na^+ conductance* and its possible role in isotonic secretion in frog skin glands is finally discussed.

Key words: Exchange diffusion — Pump and leak — Swelling-activated Cl^- channels — Shrinkage-activated Na,K,2Cl cotransport — Nonsteady-state flux ratio — Ussing chamber — Cl^- secreting epithelia

Exchange Diffusion — Low Chloride Conductance

In 1946 August Krogh delivered his famous "Croonian Lecture," in which he introduced for the first time the concept of *active transport* (pumping) of ions and *passive leakage* of ions. Ions are pumped in one direction using metabolic energy and leak passively in the other direction. It was possible to measure this transport by using radioactive isotopes that can be specifically placed on one or the other side of the membrane. This was the beginning of a major project, under the leadership of the talented young researcher Hans H. Ussing. August Krogh, Hans Hevesy and Niels Bohr had requested and obtained a cyclotron for the Niels Bohr Institute. It was the first one in Europe and it enabled the physiologists in Copenhagen to obtain the isotopes required for the project. The isotopes were $^{38}Cl^-$ and $^{24}Na^+$ and the test organisms were axolotls and isolated frog muscles and, not long thereafter, isolated frog skin, which became the object of investigation for many, many years. The work with frog muscle produced the first description of 1:1 *exchange diffusion*, which was introduced to explain the rapid exchange of Na^+ in frog muscle, where the Na^+ flux out of the muscle fiber was far too rapid to be active transport [50]. Since then, exchange diffusion has been found to be of great physiological significance, for example in red blood corpuscles, where a membrane protein exchanges Cl^- and HCO_3^-. Exchange diffusion is biologically so important because it permits the exchange of charged substances through electrically dense cell membranes without the direct use of energy. An important experimental consequence pointed out by Ussing was, of course, that steady-state isotope fluxes could not be used to measure leak

permeability or active transport, since a large portion of the flux can be exchange diffusion. Despite this caveat, in most cells it was assumed up until the early 1970's that the rapid Cl^- flux, which could be measured over the cell membrane, was due to an extremely high conductive Cl^- permeability. This also applied to the experimental system we chose to work with when I started at the August Krogh Institute, namely, Ehrlich ascites tumor cells (EATC). Up until 1975 it was generally assumed that Cl^- transport in EATC, which formed our experimental system, was due to a simple diffusion process. Inspired by Ussing, we decided to see if this was actually the case. We found that Cl^- transport was completely dominated by an exchange diffusion process [10, 19], as defined by Ussing in 1947 and found previously in red blood corpuscles (*see* [61]). Less than 5% of the Cl^- flux was a conductive leak flux, and thus, the Cl^- conductance was much lower than previously estimated from measurements of unidirectional tracer fluxes [10, 19]. Shortly thereafter, I showed that the rate constant for steady-state Cl^- flux is increased with both increasing and decreasing cell volume [12] and found that the increase in Cl^- flux after cell swelling reflects an increase in the conductive Cl^- flux, whereas the increase seen in shrunken cells with low intracellular Cl^- is due to an increase in the co-transport of anion and cations [12, 13] (*see below*).

Volume Regulation, Swelling-activated K^+ and Cl^- Channels

The pump-leak steady-state concept was introduced by Krogh in 1946 as mentioned above, but it was developed in detail by Leaf and Ussing [29, 52]. This description, along with a description by Tosteson and Hoffman appearing at virtually the same time [48] stands to this day. Since then, Ussing has also contributed significantly to the study of the dynamic and controlled changes that occur in the various leak transport paths as compensating responses to volume changes. One extremely important method of studying volumes in epithelia was introduced by MacRobbie and Ussing [33]. The setup was designed in such a way that the volume of the epithelium (height) could be followed under the microscope while, at the same time, the transepithelial potential or short-circuit current could be measured. These were the first studies of volume control in epithelia, a field that has since been taken up by various groups using methods that, in principle, are based on the same idea. The experiments were actually started to test the consequences of the two-membrane hypothesis. A very important step in this direction was Ussing and Windhager's [58] model of the epithelium as a multilayered *functional three-dimensional syncytium* with collective properties as assumed in the two-membrane hypothesis. According to this theory, the epithelial cells are connected via intercellular ion-permeable pores. This means it is meaningful to correlate volume changes in the entire epithelium with the cellular transport parameters. This *coupling* between cells was an important assumption that has since been of great significance, particularly in the study of leaky epithelia.

My own work with cellular volume regulation in EATC began when I went to work at the August Krogh Institute. In 1974 we reported that EATC were capable of volume regulation after swelling in hypotonic media, as a function of a swelling-activated K^+ conductance [9]. When working with the exchange diffusion of Cl^-, it occurred to me that since the Cl^- conductance was so low, it almost had to be swelling-activated, if the cells were to be capable of losing KCl rapidly enough to explain the rather fast volume regulation we had measured. I, subsequently, demonstrated that the Cl^- flux increased 2.5-fold at 40% cell swelling and proposed [12] that the entire increase was due to a rise in the conductive Cl^- flux which, consequently, must have risen approximately 50-fold. In a later, more direct, measurement it was found that under these conditions the Cl^- conductance is actually increased 60-fold [27]. The cell swelling-activated Cl^- channel in EATC was, in cell-attached patches, found to have a single-channel conductance of 3–7 pS [1]. In 1978 I believed I was the first ever to propose a volume-activated Cl^- conductance, but Ussing informed me that a volume-sensitive conductive Cl^- transport path had been proposed in the basolateral membrane of frog skin epithelium by MacRobbie and Ussing back in 1961 [33]. That was one of the countless times I was impressed by how early Ussing had had many of the ideas that later proved to be of great importance. Today, a volume-sensitive Cl^- channel has been found in almost all cell types in which it has been sought. One major problem in the MacRobbie and Ussing paper and in our study of the low Cl^- conductance in EATC [10, 19, 33] was, that the Cl^- distribution appeared to be quite close to equilibrium, based on earlier determination of the membrane potential, which made it difficult to set up a functional volume-regulation model. Activation of both K^+ and Cl^- permeabilities by swelling, as demonstrated in EATC [12], could lead to the observed RVD either if Cl^- ions were above equilibrium in the cells from the beginning, so that the potential was between the equilibrium potential for K^+ and Cl^-, or if the K^+ permeability rose more than the Cl^- permeability, so that the cells hyperpolarized during cell swelling, resulting in a potential between the equilibrium potentials of K^+ and Cl^-. However, the Cl^- permeability appeared to rise more than the K^+ permeability after swelling of EATC, so that it was most likely that previous potential measurements had

underestimated this. In the frog skin, it was apparently only the Cl^- permeability that rose, so that in this case it was even more necessary that the Cl^- be above equilibrium. It was not until the late eighties [27] that we were able to demonstrate with certainty in EATC that the Cl^- concentration is far above what is predicted from thermodynamic equilibrium and confirm by more direct measurements that the Cl^- conductance rose around 60-fold upon cell swelling, subsequently exceeding the K^+ conductance by a factor of 2.2. A Cl^- concentration far above what is predicted from thermodynamic equilibrium was also shown for frog skin principal cells [62]. Both in frog skin [53–55] and in Ehrlich cells [20] this was predicted by description of a volume-sensitive anion/cation cotransporter, which was running at a very low rate at normal cell volume, but activated after cell shrinkage (*see below*).

Later-described Characteristics of the Cell Swelling-activated Cl^- Current

After the activation of a conductive pathway by cell swelling was proposed in the basolateral membrane of frog skin principal cells [33] and demonstrated in EATC [12], swelling-activated channels have subsequently been demonstrated in a huge number of cell types (for references *see* [37]). The whole-cell Cl^- currents can be repetitively activated by hypotonic exposure in EATC (*see* Fig. 1). The whole-cell Cl^- currents activated by cell swelling ($I_{Cl,vol}$) are generally found to exhibit moderate outward rectification (*see* Fig. 1) and depolarization-induced inactivation [40] although this is moderate in EATC [44]. The sequence of anion permeabilities in EATC [44] and in many other cells is $SNC^- > I^- > NO_3^- > Br^- > Cl^- > F^-$ (Eisenman I sequence) [4, 8, 22, 30, 35, 39]. The unitary conductance of swelling-activated anion channels varies, but "intermediate conductance" (11–80 pS) channels appear to be the most common ones, described in many different cell types (*see* 37, 40, 41, 46]). Single-channel studies of swelling-activated "Mini" Cl^- channels (1–10 pS) have also been reported in a few cell types (*see* [17] for references). Whole-cell $I_{Cl,vol}$ in EATC was found to be Ca^{2+}-independent, insensitive to niflumic acid and relatively insensitive to DIDS, but inhibited by tamoxifen [44]. The molecular identity of the volume-sensitive Cl^- channel is still unknown.

The Cl^- channel that is activated upon swelling is a different channel than the one that is activated by an increase in $[Ca^{2+}]_i$ ($I_{Cl,Ca}$) [44], and it does not appear to be activated by membrane stretch [1]. It is a possibility that $I_{Cl,vol}$ is activated by unfolding of membrane invaginations as suggested by Okada [40], since such an unfolding during hypotonic cell swelling has been demonstrated by scanning electron microscopy in Ehrlich cells [12]. If that is the case, then the F-actin cytoskeleton could modulate the volume sensitivity of the channel by providing resistance to this unfolding. (*See* [43] for further discussion of the role of the cytoskeleton and [38, 49] for the eventual role of caveolae in this process.) We recently found that the small GTP-binding protein RhoA plays a central role in activation of $I_{Cl,vol}$, since NIH3T3 mouse fibroblast cells, which express constitutive active RhoA, activate a Cl^- current at far lower cell swelling than do the control cells, and the Cl^- current is inhibited by an Rho kinase inhibitor (K. Breisner et al., submitted).

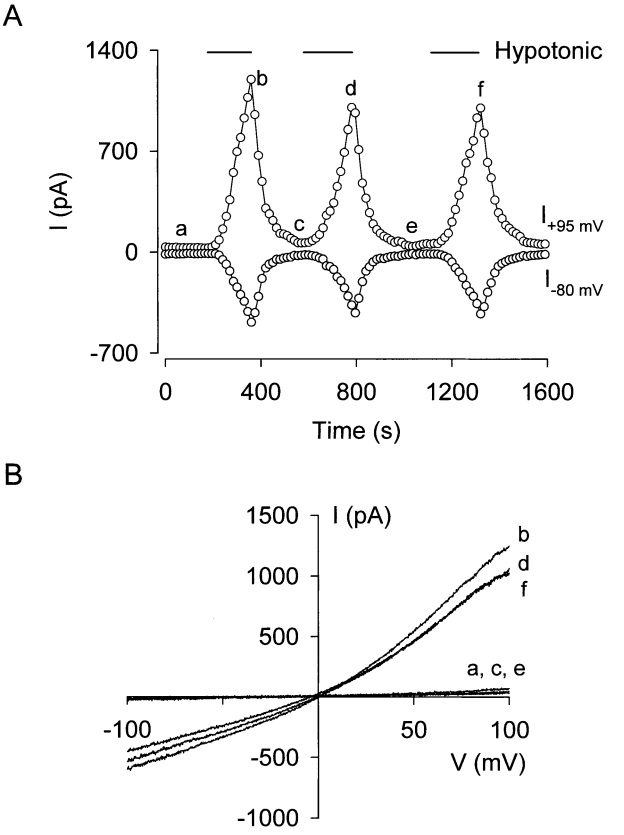

Fig. 1. Repetitive activation of an outwardly rectifying Chloride current by cell swelling. Linear ramp protocols were applied every 15 sec, and periods of hypotonic exposure were as indicated by the horizontal bars. Data points were calculated as the average current measured in a small window around 95 mV and −80 mV. The $[Ca^{2+}]_i$ was buffered at 10 nM using 10 mM EGTA. The isotonic bath solution was a modified Ringer solution containing D-mannitol (27% of the osmolarity). The 27% hypotonic solution was prepared by omitting mannitol. C. Hougaard and E.K. Hoffmann unpublished results; similar results have been published in [44].

With regard to volume regulation in the epithelial cell, Ussing returned to the subject in the 1980's and made a series of important contributions toward the elucidation of this topic [55]. During these years I constantly had the opportunity to engage in fascinating discussions with Ussing, first on shrinkage-

activated anion-cation cotransport and later on a possible regulatory mechanism for tonicity during isotonic water transport (*see below*).

Non-Steady-State Flux Ratio Analysis as a Tool for Characterizing Pathways: Shrinkage-activated Na,K,2Cl Cotransport

Studying active and passive ion transport requires a theory capable of describing electrodiffusion across a biological membrane or across an epithelium. This is difficult, since one must assume either a linear concentration gradient across the epithelium, which is not particularly likely, or a constant electric field across the epithelium, which also may be unlikely. Ussing's special intuition came to his aid in this case and he came up with the idea of not just examining the individual fluxes, but also the ratio of the influx and the outflux. He was able to show that this ratio was a function of a state that is independent of the barrier the ion passes, thus being dependent only on the electrical potential difference and the ratio of the concentrations on the two sides. He immediately began using the theory and published the results in his subsequently so famous flux-ratio article [51]. He and Hilde Levi later showed that iodide produced a flux ratio that indicated iodide was transported passively, while Na^+ exhibited a far higher flux ratio and, thus, was *transported actively* [31]. The use of isotopes and the flux-ratio equation provided an ingenious instrument for distinguishing *passive electrodiffusion* from other forms of transport.

Originally, the flux-ratio equation was derived only for steady-state fluxes. Ussing was convinced, however, that it was also valid for a great number of non-steady-state situations. The mathematical proof that he was correct was published much later, however [45]. This further expanded the usefulness of the flux-ratio equation enormously. As a typical example of the many applications of the flux-ratio analysis, I myself have used it to distinguish between NaCl and Na,K,2Cl cotransport in EATC cells. In 1981 we showed that the primary process during a regulatory volume increase (RVI) response in Ehrlich cells was activation of an otherwise quiescent bumetanide-sensitive Na,Cl, or Na,K,2Cl cotransport system, with subsequent replacement of Na^+ with K^+ via the Na/K pump [11, 13]. We were unable to determine whether it was an Na,Cl cotransport system or an Na,K,2Cl cotransport system that was involved. In 1981, when Ussing and Steen Knudsen proved that the flux ratio can be used under non-steady-state conditions as well, and that the value extrapolated to zero time should represent the true flux ratio at that time [45], we decided to utilize this to determine which system was responsible for ion uptake during RVI [20]. As an expression of the cotransport system, we measured the bumetanide-sensitive component and undertook a non-steady-state flux-ratio analysis of the unidirectional Cl^- fluxes during the RVI process. The pump was inhibited with ouabain. We found that the flux ratio for the bumetanide-sensitive unidirectional Cl^- influx and efflux was 1.85. For an electro-neutral anion-cation cotransport the ratio of products of the concentrations of the transported ions will indicate the direction of a net flux and, moreover, give a maximal value for the flux ratio. As the transport system becomes saturated the flux ratio will decrease. The analysis showed that a cotransport of K,Na,2Cl could exactly account for the flux ratio, whereas a cotransport of Na,Cl would give a much higher flux ratio, unless the cotransport system was highly saturated. In our view, two observations made the Na,K,2Cl cotransport a little unlikely, however, and that was because a net uptake of KCl was found even at low extracellular K^+ and because no K^+ uptake was found during RVI in ouabain-poisoned cells. If it was a Na,K,2Cl transport system and not a NaCl cotransport system that was activated upon shrinkage, there would have to be a rapid recycling of K^+ across the membrane during RVI. Thus, whether it was the one cotransport system or the other that was activated during shrinkage of EATC remained a somewhat open question. The main message [11, 13, 20] was that an anion-cation cotransport system was activated. At precisely that same time, Ussing [53] proposed shrinkage activation of an NaCl cotransport system in the basolateral membrane of frog skin. In [54] and [55], however, he is already relatively sure it is an activation of a Na,K,2Cl cotransporter and he suggested to me that I should finish that question for Ehrlich cells, too, by blocking a possible recycling of potassium with Ba^{2+} and then see if not only the flux-ratio analysis, but also the stoichiometry of the ions taken up in the presence of ouabain would fit with a Na,K,2Cl and not a NaCl cotransporter. I did not do that until later, after we had isolated the cotransporter on a bumetanide-sepharose affinity column [5] and had developed an antibody against the cotransporter, which could inhibit it from the outside [3]. We then finally went back to the question and made a thorough characterization of the shrinkage-activated cotransporter, including the suggested non-steady-state flux-ratio analysis in the presence of both ouabain and Ba^{2+}. Of course this was a good idea. The results showed very nicely a stoichiometry of the bumetanide-sensitive net fluxes close to 1:Na;1:K;2:C1 (*see* Table 1). Under these circumstances the K^+ and Cl^- flux ratios for the influx of the bumetanide-sensitive component were estimated at 1.34 ± 0.08 and 1.82 ± 0.15, which should be compared to the gradient for the Na,K,2Cl cotransport system at 1.75 ± 0.24 [23].

Various isoforms of Na,K,2Cl cotransporter (NKCC's) have been cloned, including the NKCC-1

Table 1. Non-steady-state flux-ratio analysis of unidirectional chloride and potassium fluxes during Regulatory Volume Increase in Ehrlich cells

Bumetanide-sensitive component	Cl^-	K^+	Na^+
		$\mu mol \cdot g\ dry\ wt^{-1} \cdot min^{-1}$	
Net flux	29 ± 3.8	12 ± 2.6	15 ± 1.9
Unidirectional influx	66 ± 6.1	46 ± 2.7	–
Unidirectional efflux	37 ± 4.1	34 ± 1.4	–
Flux ratio Influx/efflux[a]	1.8 ± 0.15	1.3 ± 0.08	–
Ion concentration ratio: $\frac{[Na]_o[K]_o[Cl]_o^2}{[Na]_i[K]_i[Cl]_i^2}$		1.8 ± 0.24	
Stoichiometry of net fluxes: $[Cl^-]:[K^+]:[Na^+]$	2.0	0.8	1.0

The bumetanide-sensitive components were determined as the difference between the fluxes in parallel groups with and without 30 μM bumetanide.

The values are given as mean ± SEM for five sets of individual experiments, with concurrent measurements of unidirectional ^{42}K and ^{36}Cl influx and net ion uptake. The mean value of the measured internal ion concentrations were (mmol/liter cell water): K^+, 182 ± 13(5); Na^+, 20 ± 1 (5); Cl^-, 67 ± 6 (n = 5). The mean value of the measured external ion concentrations were (mM): K^+, 5.9 ± 0.2 (n = 5); Na^+, 184 ± 8 (n = 5), Cl^-; 156 ± 6 (n = 5).

[a] The values are given as the mean ± SEM of the flux ratios calculated in each individual experiment.(from [23]; similar flux ratio analysis for Cl^- in [20]).

and NKCC-2 subgroups. The NKCC-1 isoforms are found in the basolateral membrane of epithelia, e.g., the shark rectal gland cotransporter [63] and two mammalian homologues [2, 42]. The NKCC involved in RVI in EATC has more than 90% homology with the epithelial NKCC-1 isoform from mouse kidney (B.S Jensen and E.K. Hoffmann, unpublished results).

Activation of the Cotransporter

In the article just mentioned above [23], we showed that the cotransporter could also be activated after addition of bradykinin, which induces inositol phosphate-mediated Ca^{2+} signaling, resulting in KCl loss and cell shrinkage. The same is found with histamine and thrombin [18]. In this case, cotransport activation could be due to an increase in cellular Ca^{2+} (direct or indirect), the drop in cellular Cl^- activity, or cell shrinkage. When cell shrinkage was prevented by making the flux medium a K^+ equilibrium salt solution (KCl substituted for NaCl), activation of the cotransporter essentially disappeared, although a small and transient activation within the first minute was still seen [14]. This indicates that cell shrinkage or a decrease in cellular Cl^- activity is a prerequisite for a pronounced and persistent activation of the cotransporter. However, the experiment cannot distinguish between the drop in cellular Cl^- activity and cell shrinkage. This problem is identical in secretory epithelia. The fact that both a drop in intracellular Cl^- activity and cell shrinkage influenced activation of the cotransport system was first proposed by Ussing [53] and that a reduced intracellular Cl^- concentration could play a permissive role for the activation of the cotransporter was subsequently discussed for Ehrlich cells [20] and for thymocytes [7]. The importance of cell shrinkage versus the drop in cytosolic Cl^- activity in secretory epithelia has remained unclear, but it has been proposed that the key event is the fall in cytosolic Cl^- activity, not the shrinkage [32]. We returned to this problem again recently when studying Cl^- secretion in the operculum epithelium from *Fundulus* and in cooperation with Rainar Greger's group also in the rectal gland of *Squalus acanthias* [6] (*see* next section).

NKCC-1 in EATC is activated after cell shrinkage via activation of myosin light chain kinase (MLCK) and phosphorylation of the myosin light chain (MLC). The shrinkage-induced NKCC activation is strongly inhibited by the MLCK inhibitor ML-7 in EATC [26]. The cytoskeleton appears to play an important role in regulation. Thus, cell shrinkage is found to result in actin polymerization and this appears to be required for activation of NKCC-1 under hypertonic conditions, just as an intact cytoskeleton is necessary for keeping the NKCC-1 in a silent state under isotonicity. F-actin depolymerization results in partial activation of NKCC-1 even under isotonic conditions [24, 34]. In EATC, cell shrinkage activates NKCC-1 much more potently than does exposure to cytochalasin or cell swelling [24], and it is tempting to suggest a three-state situation may exist: (i) a silent state where the NKCC-1 is inactive, (ii) a partly activated state where contact with the cytoskeleton is disrupted, and (iii) an activated state, initiated by cell shrinkage in an F-actin-dependent manner, with no possibility of going from

the partly activated state to the activated state. In agreement with this notion, in isolated EATC blebs, which are devoid of F-actin and myosin II [36], NKCC-1 is permanently in a partly activated state [16], it cannot be further activated by cell shrinkage and ML-7 has no effect on the NKCC activity (E.K. Hoffmann, unpublished).

Short-Circuit Setup, the Ussing Chamber. Cl- Secreting Epithelia

When frog skin is mounted between two identical salt solutions, the inside of the skin, or the side facing the blood, becomes positive (~100 millivolts). Ussing's hypothesis was that active pumping of Na^+ ions across the frog skin created the potential difference and that Cl^- ions followed passively along. The problem, however, was to demonstrate this with certainty. Ussing's idea was simple and ingenious. He would short-circuit the frog skin or, stated differently, he would set the electrical potential across the frog skin to zero. If the concentration of an ion is the same on both sides of the epithelium and the electrical potential difference is zero, there is no force that can produce a net ion transport and the flux ratio will be unity. If an ion is transported actively, it must have a flux ratio that is higher. Ussing and K. Zerahn used this fact to construct the *Ussing chamber* that later became so famous and conducted the first *short-circuit experiments* [59]. They did, indeed, show that only Na^+ was actively transported, while Cl^- followed passively along, and that the short-circuit current was identical to the Na^+ transport. At the same time, the oxygen consumption by the preparation increased, showing that energy was used for the Na^+ transport. Finally, the experiments showed that if the formation of ATP was blocked with dinitrophenol, then the Na^+ pump stopped, indicating that ATP must be used in some way or another for active transport. This short-circuit experiment (the *Ussing chamber technique*) was a breakthrough in the study of membrane transport. In the years following Ussing's presentation of his results at a congress in Copenhagen in 1950, his laboratory became a Mecca for foreign researchers who wanted to study transport across membranes under Ussing's tutelage. Combined with isotope techniques using different isotopes on the two sides and measuring the flux ratio, the method (*see* Fig. 2) made it possible to study steady-state transport paths in a way that had never been possible before. The short-circuit technique is still used in hundreds of laboratories around the world to study ion transport across epithelia. It was found that in other tissues it was the Cl^- ion that produced the current across the epithelium, building up the potential, which was also the case in the glands of frog skin, when stimulated with adrenaline [25].

Fig. 2. Short-circuited isolated frog skin in an Ussing chamber. Hans H. Ussing and his technical assistant through many years, Birgit Hasman, Copenhagen 1966.

Much later, I had an opportunity to study such a Cl^--secreting epithelium when, in the summers of 1998 and 1999 at Mount Desert Island Biological Laboratory, Professor José Zadunaisky, Erik Hoffmann and I studied Cl^- transport across the operculum epithelium of the little eurohaline fish *Fundulus heteroclitus*. The epithelia were mounted in small Ussing chambers constructed by Zadunaisky (*see* [64]) and the experiments were standard short-circuit current experiments. That gave me a good opportunity to read up on the classic Ussing articles, which was a pleasure. For example, we returned to the question of whether the key process in the activation of NKCC after activation of secretion is cell shrinkage or a fall in cytosolic Cl^- activity. We were able to show that if we stimulated Cl^- transport with a β-agonist such as isoproterenol (10^{-5} M), but at the same time made the medium weakly hypotonic, then all stimulation disappeared (*see* Fig. 3), Under these conditions, the cytosolic Cl^- activity drops, but the cells do not shrink. We interpreted this to mean that a certain amount of cell shrinkage was necessary to activate the basolateral Na,K,2Cl cotransporter, a result that fit in well with Ussing's studies on the activation of the Na,K,2Cl cotransport system after cell shrinkage in frog skin epithelium (*see*, for example, [54]). It should be mentioned, as discussed in

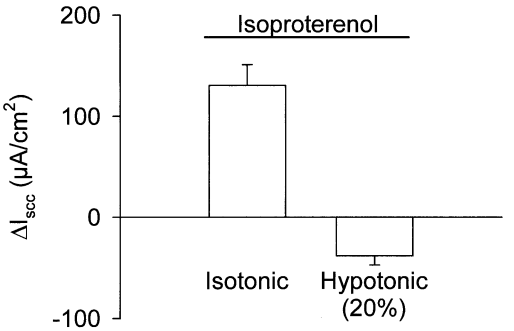

Fig. 3. Change in short-circuit current of an operculum epithelium from *Fundulus heteroclitus* mounted in a modified Ussing chamber with regular teleost Ringer's solutions on both sides. The short-circuit current was used as a measure of the Cl^- current, *see* [64] for method details. Isoproterenol (10^{-5}M) was added with or without simultaneous addition of 20% distilled water and the short-circuit current followed with time. The change in current is calculated as the difference between the current before addition of the agonist and the current after a new steady state is attained. The addition of isoproterenol alone stimulated the short-circuit current up to 258 ± 10 μA/cm^2 [15]. E.K. Hoffmann, E. Hoffmann and J. Zadunaisky, unpublished results.

[65], that a decrease in current could also result from an eventual swelling-activation of a basolateral Cl^- channel. The same issue was examined in isolated perfused rectal gland tubules next door in a collaboration between me and professor Rainar Greger's group. Also there we could conclude that the primary and key process that triggers Na,K,2Cl cotransporter activation is a transient cell shrinkage [6].

Isotonic Water Transport — Shrinkage-activated Sodium Conductance

During the late 1980's, Ussing began his study of *isotonic water transport* [56], a topic that occupied Ussing's thoughts and ideas until his death in December 2000. It was only recently that the simple and ingenious idea that it was the regulated "recycling" of the actively pumped ion (the Na^+ ion) that controlled the tonicity of the transported fluid, received a theoretical treatment (*see* [28]). When Ussing and I wrote a survey article together [21], the section we discussed most intensely was the one entitled "The Role of Cellular Volume Regulation in Isotonic Water Transport." There we presented the hypothesis that the secretion is kept isotonic because the recirculation of Na^+ is regulated by the osmolarity of the secreted fluid (*see* Fig.4). How does this work? Well, the idea was that "the apical Cl^- and Na^+ channels in the gland cells react to volume changes in the same way as those of EATC: cell swelling increases Cl^- permeability and reduces Na^+ permeability, whereas shrinkage has the opposite effect. Let us now assume that the secretion is becoming too hypotonic: the cells swell and more Cl^- is secreted, whereas less Na^+ is recycled (Fig.4, upper panel)" [21]. Thus, more NaCl is secreted, making the lysate, more hypertonic again. If the lysate has become too hypertonic, then the cell shrinks, the Cl^- channel closes, the Na^+ channel opens, and a greater part of the Na^+ ions recirculate. Thus, less NaCl is excreted, decreasing the tonicity of the transported fluid. It is tempting to suggest that this is a situation where the fact that Cl^- and Na^+ channels are oppositely regulated by changes in cell volume could play an important physiological role. Our only background for proposing such a volume-sensitive Na^+ channel in the apical membrane was that I had described a shrinkage-activated conductive Na^+ permeability in Ehrlich cells [12], thus, there could perhaps also be one in the epithelial cells with such a key function. It should be pointed out here that the model is not actually dependent on the existence of a volume-sensitive Cl^- channel in the apical membrane. Such a channel will reinforce the effect, but what is necessary and sufficient in the model is an apical Na^+ channel that is shrinkage-activated, and the assumption that the apical membrane of the gland cell is much more permeable to water than the basal membrane, so that the cell volume can be determined by the osmolarity of the secreted fluid. Obviously, the model is completely dependent on the presence of an apical entrance mechanism for Na^+, as for example Na^+ channels. Sørensen and Larsen [47] have demonstrated co-existence of Na^+, Cl^- and K^+ channels in the luminal membrane of exocrine glands by patch-clamping the luminal membrane. These Na^+ channels had a conductance of 5pS at the spontaneous membrane potential. The selectivity of the channels was not studied. Shrinkage-activated Na^+ channels have been described in several cell types [60]. It should be added that the above description, of course, does not attempt at all to explain how the paracellular fluid transport in the outward direction is actually accomplished—that is another discussion.

Whole-cell Patch-clamp Experiments in Ehrlich Cells—Shrinkage-Activated Cation Channels

We recently resumed our study of the shrinkage-activated Na^+ channel in whole-cell patch-clamp studies with Ehrlich Lettre cells. The Ehrlich Lettre cells definitely have a cation conductance activated by cell shrinkage. This conductance is blocked by Na^+ channel blockers (concentrations in the high μmolar range) with the following order of potency: EIPA $<<$ amiloride $<$ gadolinium $\sim$ benzamil (P. Lawon, C Hougaard, E.K. Hoffmann and F.Wehner, unpublished). Its selectivity for sodium remains to be tested, however. Whether the apical Na^+ channels in the glandular cells of frog skin will prove to be activated upon shrinkage has not yet been studied.

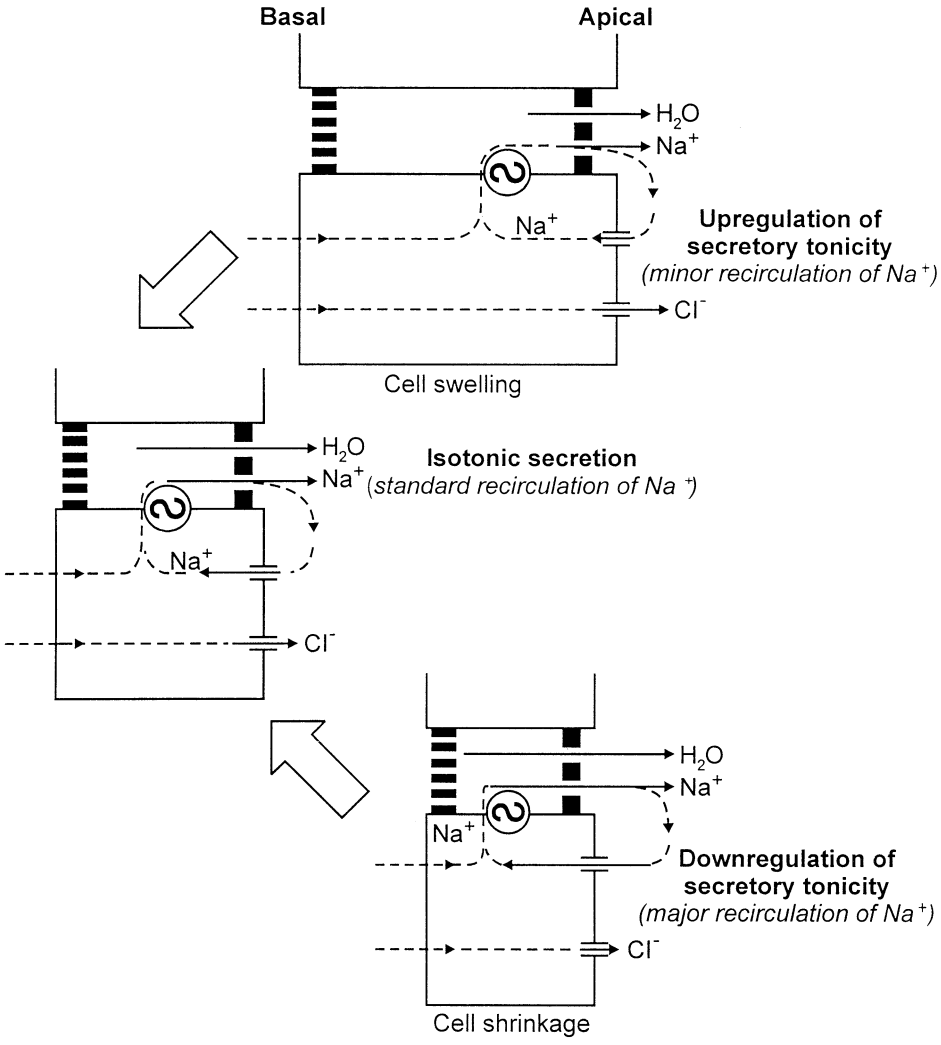

Fig. 4. "Feedback regulation of the tonicity of the frog skin gland secretion. It is assumed that the apical membrane of the gland cells is much more permeable to water than the basal membrane, so that the cell volume is determined by the osmolarity of the secretion. The *middle panel* (stationary isotonic secretion) shows the cell volume when the secretion is isotonic. If the secreted fluid attains a lower tonicity (*upper panel*), the cells will swell. This elicits a regulatory volume decrease (RVD) response (increased chloride permeability and decreased Na^+ recycling), i.e., increased tonicity of the secretion. Conversely, if the tonicity of the secretion should become too high (*lower panel*), the cells shrink, and we see a regulatory volume increase response , with reduced Cl^- transport and an increased recycling of Na^+, i.e., reduced electrolyte secretion" (from [21]), the net results being a reduced tonicity of the secretion. Reproduced from [21] with the minor modifications that the Na^+/K^+ pump is placed in the lateral membrane and a barrier is shown at the level of the basement membrane preventing the major fraction of the pump-generated Na^+ flux from returning to the serosal bath (*see* [57]). For details in the pathways for the three ions involved, *see* [47].

Conclusion

With clear analysis and a lot of common sense, there is no problem that cannot be solved—this was the doctrine the young Ussing learned. Ussing became a researcher with such a sure sense of the simple solutions to problems that nature simply should have chosen them. Ussing's theories were frequently met with skepticism immediately after they were published. Nevertheless, he always turned out to be correct. We are many who have been inspired by our contact with such a great researcher, and to our best ability we have strived to live up to his example.

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