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Review

Hans H. Ussing—scientific work: contemporary significance and perspectives

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Abstract

As a zoologist, Hans H. Ussing began his scientific career by studying the marine plankton fauna in East Greenland. This brought him in contact with August Krogh at the time George de Hevesy, Niels Bohr and Krogh planned the application of artificial radioactive isotopes for studying the dynamic state of the living organism. Following his studies of protein turnover of body tissues with deuterium-labeled amino acids, Ussing initiated a new era of studies of transport across epithelial membranes. Theoretical difficulties in the interpretation of tracer fluxes resulted in novel concepts such as exchange diffusion, unidirectional fluxes, flux-ratio equation, and solvent drag. Combining methods of biophysics with radioactive isotope technology, Ussing introduced and defined the phrases 'short-circuit current', 'active transport pathway' and 'shunt pathway', and with frog skin as experimental model, he unambiguously proved active transport of sodium ions. Conceived in his electric circuit analogue of frog skin, Ussing associated transepithelial ion fluxes with the hitherto puzzling 'bioelectric potentials'. The two-membrane hypothesis of frog skin initiated the study of epithelial transport at the cellular level and raised new questions about cellular mechanisms of actions of hormones and drugs. His theoretical treatment of osmotic water fluxes versus fluxes of deuterium labeled water resulted in the discovery of epithelial water channels. His discovery of paracellular transport in frog skin bridged studies of high and low resistance epithelia and generalized the description of epithelial transport. He devoted the last decade of his scientific life to solute-coupled water transport. He introduced the sodium recirculation theory of isotonic transport, and in an experimental study, he obtained the evidence for recirculation of sodium ions in toad small intestine. In penetrating analyses of essential aspects of epithelial membrane transport, Ussing provided insights of general applicability and powerful analytical methods for the study of intestine, kidney, respiratory epithelia, and exocrine glands—of equal importance to biology and medicine.

Keywords: Exchange diffusion; Sodium pump; Two-membrane hypothesis; Solvent drag; Isotonic transport; Sodium recirculation; Obituary; Science history

1. Introduction

Hans Henriksen Ussing's death in December, 2000 marks the ending of a 70-year-long era, which began in Copenhagen with the introduction of artificial radioactive tracers to biology by George de Hevesy and August Krogh. Ussing's investigations of active sodium transport by frog skin and the ion permeability of this preparation were epoch-making. His methods and experimental analyses founded the theories of epithelial bioelectricity and transport physiology, which constitute corner stones of membrane biology.

At an age of 17 years, after finishing high school in 1929, Ussing (Fig. 1) was enrolled as a student of Natural History and Geography at the University of Copenhagen. He obtained his Master's Degree with first class *cum laude* in 1934. After a short period as scientific assistant in the laboratory of August Krogh, Ussing became Lecturer in Biochemistry, and in 1951 he became extraordinary Professor of Zoophysiology. During the period from 1958 to 1981, he was ordinary Professor and Head of Biochemistry.

Ussing's scientific work is divided between three entirely different fields: marine biology, biochemistry, and physiology. Although never in touch with each other, there are logical reasons for his move from one field to another.

2. Marine biology and East Greenland's marine zooplankton

"The Three-Years Expedition to the Christian Xth Land 1931–34" headed by Danish geologist Lauge Koch was a

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Fig. 1. Hans Henriksen Ussing, 30 December 1911–22 December 2000. The photo was taken in 1955 at the time he became Fellow of the Royal Danish Academy of Sciences and Letters. Courtesy of the Academy.

truly 'big-science' project aiming at an overall description of the large and not very accessible area between 71° and 75° Northern latitude. Two specially designed laboratory vessels, "Gustav Holm" and "Godthaab", echo sounders and airplanes were at the command of scientists covering hydrography, geology, botany, zoology, geography, and ethnography. Officially, the expedition was sent out from Copenhagen by the Danish Prime Minister Thorvald Stauning for exploring new settlement possibilities for Eskimos and for finding new mining localities. There was, however, also a hidden political agenda as in 1931 Norway had declared a large area of East Greenland occupied under the name 'Erik Raude's Land' (i.e., Erik The Red's Land). As an able field biologist and a specialist in Crustaceans, Hans Ussing was invited to participate as a student on the 'summer team' in 1933 on this peaceful Danish flotilla. He would collect zooplankton and hydrographical data on temperature and salinity by vertical pulls from 50 and 25 m within the fjords around Scoresby Sound. Summer teams of small groups of scientists arrived when the fjords became ice-free and they returned to Copenhagen early autumn. The summer teams were followed by the 'winter teams', who collected samples during the long polar darkness in icy coldness and frequently also during terrible snowstorms. Thereby, plankton and hydrographical data were collected on selected positions during the whole year: the winter teams from a heated temporary wooden cabin around a drilled hole in the ice cover, and the summer teams from the expedition vessel "Godthaab". The winter teams brought home about 500 plankton pulls from Ella Island and 40 pulls from Eskimonæs. After returning to Copenhagen in 1933, all of the zooplankton samples, i.e., both those collected during summer by Hans Ussing and during winter by others, were handed over to Ussing whose task it was to determine the species of the zooplankton, and analyse its biological significance for the ecology of the fjords of East Greenland. Taxonomy, reproduction, nutrition, and vertical distribution of the zooplankton during 2 year's cycles were analyzed and published in Ussing's 100-pages-long doctoral thesis defended in 1938: "The biology of some important plankton animals in the fjords of East Greenland" [1]. Highly recognized by his two opponents Ragnar Spärck, Professor of Zoology, and August Krogh, Professor of Zoophysiology, this was the first description and biological analysis of an arctic plankton fauna.

3. Ussing's first visit to August Krogh's laboratory

The oral defense was not the first time Krogh and Ussing were facing each other. Since 1934, Ussing had been member of Krogh's group of young scientists. Copepods, which Ussing had become acquainted with during his zoology study, constituted the dominating animal group of the relatively species-poor plankton collections from East Greenland. But the samples—especially of larvae—were not always in a state that allowed precise species identification. Concerned about this difficulty, Ussing got the idea of raising antibodies against selected organisms for singling out the larvae species by species for mass calculations. He decided to consult August Krogh for his advise on how to proceed with this brilliant idea. According to Ussing [2], Krogh did not have the clue. Instead, he turned the conversation towards his own interests by telling Ussing about his recent visit to Urey's laboratory at Columbia University in USA. In 1931, Harold C. Urey discovered heavy hydrogen (D) and shortly after, in 1932, he identified heavy water, D_2O [3]. From him, Krogh had received a sample of heavy water, which he was now planning to use for physiological studies in Copenhagen [4]. Impressed by the young Ussing's innovative thinking, Krogh offered Ussing a position as his assistant. Thus, as it turned out, Ussing now entered the second 'big science' project in the 1930s Copenhagen [5].

About the time of Ussing's meeting with August Krogh, Niels Bohr and George de Hevesy had invited Krogh to cowork on application of isotope tracers in studies of the dynamic state of living cells (Fig. 2). It was Hevesy's brilliant idea to use radioactive isotopes for measuring the rate of biological processes. He had convincingly demonstrated the method's superior possibilities in a study from 1923 in which he applied natural radioactive lead, the thorium B isotope, for investigating turnover of lead in *Vicia faber* [6]. In 1934, Frédéric Joliot and Irène Curie by α-particle bombardment of aluminum and boron succeeded in producing unstable isotopes of phosphorous and nitrogen, respectively, which decayed by emission of positrons [7]. This was the first description of artificial radioactive elements and Hevesy immediately realized the exciting possibilities for biologists by applying artificial isotopes as tracers

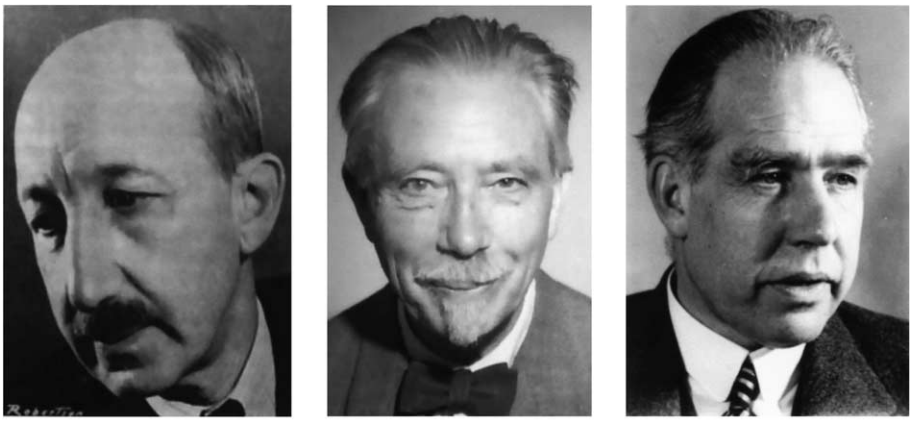


Fig. 2. Artificial radioisotopes were introduced in biological research in the 1930s Copenhagen. Hevesy conceived the idea and discussed it with Bohr and Krogh. Their applications to the Rockefeller Foundation resulted in grants for building a cyclotron at the Institute for Theoretical Physics, University of Copenhagen, and for material and further equipment. Also, the Carlsberg Foundation supported this large-scale collaboration between physicists and biologists. Ernest O. Lawrence in Berkeley designed the first cyclotron and his laboratory assisted the team of Danish physicists and engineers in building the cyclotron in Copenhagen, which became the first in Europe. *George de Hevesy*, courtesy Hilde Levi. *August Krogh*, August Krogh Institute photo archive. *Niels Bohr*, courtesy Niels Bohr Archive in Copenhagen.

[8,9]. With isotopes, exchanges of molecules could be studied without perturbing the state of the tissue under investigation. This new tool would also enable Krogh to return to the problem pointed out to him by Christian Bohr[1] about 40 years earlier, whether transport of molecules through living membranes may be driven by 'vital' forces, that is, forces derived from energy metabolism of living cells. The discussions in Copenhagen between Bohr, Hevesy and Krogh resulted in an application to the Rockefeller Foundation in New York for building Europe's first cyclotron. A large sum of money was granted and the first radioactive isotopes were produced towards the end of 1938 [5]. Ussing, however, made a detour by application of deuterium in biochemical studies, before he applied radioactive isotopes of small ions for permeability studies on biological membranes.

4. Biochemistry: protein turnover and handling of amino acids by kidney

In 1935, Krogh, Hevesy and Hevesy's collaborator, Hofer [10], used heavy water for measuring the water permeability of frog skin. Their experiments showed the D_2O permeability to be independent of the direction of the flux of heavy water, thus confirming their hypothesis that water moves passively, i.e., by diffusion, through biological membranes. To their surprise, however, the osmotic permeability of ordinary water turned out to be about five times larger than the D_2O-permeability. As they were unable to suggest a consistent explanation they forwarded the depressing conclusion that heavy water could not be used for permeability studies. Seventeen years later, Ussing himself attacked this problem and solved it, which I shall return to below.

Since Krogh could no more recommend continuing of the permeability studies, Ussing decided to attempt incorporating deuterium into amino acids. If successful, he would have a most powerful tool for exploring whether proteins are turned over in nongrowing tissues. After discussions with Kaj Linderstrøm-Lang at the Carlsberg Laboratory, Ussing succeeded in incorporating heavy hydrogen in the α-position of amino acids by heating them in sulfuric acid. His subsequent experiments with an adult rat showed that during a 3-day feeding period, the liver proteins contained deuterium corresponding to a 10% replacement of nonlabeled with labeled amino acids. The number for muscle proteins was somewhat smaller, about 2.5%. This result, published in *Nature* in 1938 [11], provided the first evidence that body proteins are constantly synthesized and degraded in such a way that amino acids taken up via food are incorporated into new protein molecules, while at the same time others are catabolized. With his new tool, Ussing had demonstrated how tracer technology provides fundamentally new opportunities for exploring the dynamic state of living cells.

During these years, studies with labeled substances were introduced also in laboratories abroad for investigating the intermediary metabolism of animals. Thus, in a series of papers using D-labeled compounds, Schoenheimer, Rittenberg and their collaborators studied the metabolism of lipids of the organism, e.g., Ref. [12], and shortly after, Ussing's *Nature* paper [11] work from their laboratory showed that

[1] Christian Bohr (1855–1911) was Professor of Physiology at the Medical Faculty of the University of Copenhagen.

the heavy isotopes of double labeled dietary $D^{15}N$-leucine became incorporated into body proteins of rats in body-mass balance [13]. Ussing estimated the turnover rates in adult animals in protein balance in a subsequent study in which he—with a novel protocol—attempted to keep a constant D_2O concentration of the extracellular fluid. In the paper [14], he emphasized that the correct interpretation of this type of experiments presupposes that one can distinguish the physical and chemical compartments in which the isotope is distributed for measuring their time course of deuterium turnover. This, of course, is not possible. With the mono-exponential uptake of deuterium by proteins of different tissues taken to be an indication of the biological half times at 'dynamic equilibrium', Ussing could conclude that the turnover of hemoglobin and myosin is significantly slower than that of liver proteins and other muscle proteins. These finding have been verified in all subsequent studies.

Hitler's occupation of Norway and the bombing of Norsk Hydro's plant in Rjukan by a group of young Norwegians effectively stopped further experiments with heavy water in Copenhagen. Ussing therefore had to formulate new problems to be attacked without the use of deuterium labeled amino acids. In his subsequent and—as it turned out—last studies of amino acid and protein metabolism, Ussing enters a new and perspective-rich field of research, which he discussed with Poul Brandt Rehberg[2]. In his studies published in 1926, Rehberg could conclude that the glomeruli of human kidneys (his own!) filter about 180 liters of fluid per day—the major fraction of which is reabsorbed by the tubules, so that the delivered urine amounts to 1–1.5 l per day, i.e., less than 1% of the amount filtered [15]. As the concentration of amino acids in the ultrafiltrate and plasma would be about the same, and as amino acids normally are excreted in small amounts, Ussing safely could assume that amino acids to a very significant extent had to be transported back into blood plasma. Based on these considerations, he now asked whether the 20 amino acids are handled by one and the same mechanism or by different mechanisms. He designed methods for quantitative identification of glycine and a few other amino acids in plasma and urine, and showed that plasma concentration of glycine, as well as excretion of glycine by human kidney, are significantly increased in response to a selectively increased oral intake of this amino acid. As the excretion of the other amino acids was maintained at the same low level as that of fasting individuals, Ussing concluded that the amino acids are reabsorbed by separate mechanisms along the kidney tubule [16][3].

[2] Poul Brandt Rehberg (1895–1989) became Professor of Zoophysiology after Krogh's retirement in 1946.

[3] In his discussion of amino acid reabsorption, Ussing considers protein turnover within the tubule cells rather than membrane mechanisms [16].

5. Ussing enters the field of membrane permeability: exchange diffusion

During these years, Ussing and Krogh had many mutually rewarding discussions, but their research followed quite different tracks. While Ussing was occupied by biochemical problems, Krogh maintained his interest in the permeability of biological membranes. For elucidating the question of active transport, Krogh in 1937 showed that salt-depleted frogs take up chloride ions against steep concentration gradients [17]. Subsequently, he asked the more general question whether living organisms invest metabolic energy in creating and maintaining ion concentration gradients across cell membranes and, in freshwater animals, across the body surface. It was Krogh's working hypothesis, published after comprehensive literature studies [18], that a 'steady state' is maintained by a balance between passive and active fluxes. With the cyclotron at Niels Bohr's institute producing radioisotopes of small diffusible ions, Krogh had excellent possibilities for testing his hypothesis. However, because of the Nazis' occupation of Denmark, in 1944 he was advised to flee to Sweden [4]. Rather than abandoning his scientific research plans, Krogh turned to Ussing and asked him to shift entirely his scientific focus and take over the leadership of the projects with radioisotopes.

Ussing accepted, but he has admitted that he reluctantly left his own biochemical projects, which now had gained momentum [19]. Krogh handed over to Ussing a 'Memorandum concerning the use of isotopes for determination of ion permeabilities of cell surfaces and living membranes generally' prepared for a grant application to the Rockefeller Foundation. Typically for Krogh, he did not consider the possibility of choosing a particular biological system/ion as model for his studies, but suggested in some detail how studies of a number of different organisms (*Astacus*, *Erioheir*, the larval stage of chironomides), tissues (rabbit and rat red cells, freshwater gills, heart and striated leg muscle of the frog, tail papillae of mosquito larvae, etc.), and elements (potassium, sodium, chloride, bromide) should be carried out. In some detail, he also discussed technical problems and suggested how to solve them (or whom to consult!). In retrospect, one may say, typically for Ussing, he made his own choice. However, this is not quite true. Ussing followed Krogh's suggestion of studying the frog muscle, but while Krogh emphasized potassium, as he expected this ion to be actively transported, Ussing decided to go for the sodium ion. He realized in tracer wash out experiments the recirculation of $^{42}K^+$ between muscle cells and the interstitial fluid of low potassium concentration was not easily corrected for. He also told me he was not convinced that the putative active component of the K^+ influx could be identified as the large negative intracellular electrical potential might account for the high intracellular potassium concentration. For these reasons, Ussing decided to study transport of Na^+ in frog isolated leg muscle. A mechanism generating active sodium fluxes had been suggested by others, and Dean had named it

'the sodium pump' [20]. The experimental proof of its existence, however, was never carried out. Furthermore, the mere idea of active cation transports was most controversial. Ussing liked to recall that when he applied the Carlsberg Foundation for supporting his studies on active transport, the Chairman, Niels Bjerrum, called Ussing to his office to inform him that he was granted the money so that he himself could prove his hypothesis wrong [21].

Ussing faced significantly more difficult problems till he became the first to prove active transport of sodium in a study that stopped further discussions of this controversial issue and directed the research into a new profitable period. Ussing, with Hevesy's former collaborator, Hilde Levi, studied the efflux of sodium in isolated frog sartorius muscle with $^{24}Na^{+}$ as tracer [22]. The experimentally determined membrane flux, which took place against concentration- and electrical gradients that could be estimated, turned out to be so large that the pumping of sodium would consume most of the energy production of the muscle cells. This improbable result Ussing decided to interpret as a paradox that could be solved only if he introduced a new concept, which he denoted exchange-diffusion [23]. He defined this as a membrane mechanism that couples influx and efflux in a 1:1 proportion. As the thermodynamic work is zero, the exchange fluxes do not require expenditure of metabolic energy. We now know that exchange-diffusion mechanisms belong to the common repertoire of membrane transport systems of general physiological significance. Also, other ions are submitted to forced exchanges, and the exchange of different ion pairs is the result of the activity of highly specialized membrane proteins. To Ussing, it became clear that the muscle preparation had to be abandoned in studies of the putative active sodium transport.

6. Frog skin and the theoretical interpretation of tracer fluxes: the flux-ratio equation

In addition to the complications due to exchange-diffusion, Ussing realized that if he did not know exactly the ion activities on either side of the membrane, as well as the membrane potential, he would never be able to carry out the scientific proof of active sodium transport. Ussing's group at the Zoophysiological Laboratory consisted of Hilde Levi and Carl Christian Barker Jørgensen, and in a study from 1946 with $^{24}Na^{+}$ and $^{38}Cl^{-}$, they showed that axolotls, which are not salt-depleted, take up NaCl through the skin [24]. This finding was novel as compared to Krogh's studies, which had shown that freshwater animals in negative salt balance take up NaCl via the body surface. Isotopes were not yet available to Krogh; therefore, he had to perturb the animal's ion balance for demonstrating uptake of ions. By comparing these two studies, from 1937 [17] and 1946 [24], respectively, our students are easily convinced of the revolutionary possibilities the isotope tracer method offered physiological research. Thus, the isolated skin would be the ideal preparation for advancing the studies. As the skin of frogs was more easily isolated, it became Ussing's preferred preparation during the following 50 years.

With the isolated frog skin, only the practical problems were solved. The theoretical interpretation of tracer fluxes was not yet worked out in detail. The Nernst–Planck equation provides a useful starting point for a theoretical treatment of membrane fluxes. However, its mathematical integration through the membrane in the direction of transport was not possible due to ignorance about the variation with distance from the boundary of ion mobility, ion activity, and electrical potential. As usual, during these years, Ussing went to Linderstrøm-Lang at the Carlsberg Laboratory for discussions [25]. They agreed to consider independently an approach. When they met again the following week, they had arrived at two different mathematical solutions. Both had assumed constant mobility within the membrane, but while Linderstrøm-Lang assumed a linear concentration profile, Ussing assumed constant electrical field[4]. For a composite membrane, none of the assumptions were acceptable. Thus, urged to find another way of thinking, Ussing introduced and defined the concept of unidirectional fluxes, which he denoted 'influx' and 'outflux', respectively. By considering the above-mentioned two solutions for net fluxes, Ussing realized that the ratio of the two new variables (influx/outflux) would depend only on the electrical potential difference between the solutions bathing the two sides of the 'membrane' and their ion activities. The equation, which is now known as the Ussing flux ratio equation, was derived in the paper of 1949 with the telling title: "The distinction by means of tracers between active transport and diffusion" [27]. Considering 'appearing fluxes' Ussing's equation still holds if the membrane contains a stationary source, or sink [28].

Sten-Knudsen and Ussing [29] later proved that the flux-ratio of an ion that passes a composite plane membrane along one and only one pathway is time-independent provided appearing fluxes are considered, which is true for any combination of driving forces. According to this theorem, Ussing's original equation constitutes the special case of stationary unidirectional fluxes governed by electrodiffusion. Further to this, Bass and Carslaw [30] showed that the generalized equation applies also for fluxes into and out of a hollow cylinder, and for the case of temporary capture of the tracer at fixed sites within the composite membrane [31]. The operational significance of this class of theorems is if the experimental flux-ratio is time-dependent, the unidirectional fluxes pass through parallel pathways of different properties, which can be analysed [32–34]. A special deviation from the

[4] Ussing's derivation was published in Ref. [22], i.e., after Goldman's [26] paper from 1944. Ussing told me, shortly after his own theoretical treatment and during a visit to USA, that Kenneth Cole kindly informed him about Goldman's work, which to Ussing's embarrassment he himself had ignored. Due to the war, scientific periodicals printed overseas were not available in Copenhagen.

original equation was treated by Hodgkin and Keynes [35], who showed that under nonequilibrium conditions, transport in "single file" through a narrow pore results in a flux-ratio exponent that reflects the number of ions, which simultaneously occupy the pore.

Flux-ratio analyses with isotope tracers, therefore, have turned out to be remarkably powerful. It should be noted that the flux-ratio equation for steady state electrodiffusion was discovered already by Behn [36] and independently by Teorell [37]. They considered the ratio of fluxes of two ions of different identity but with similar charge (i.e., a "net flux ratio" [36] or "relative fluxes" [37]), wherefore the ratio of the two ions' mobility in the (homogenous) membrane entered the right-hand site of their equation. However—without considering isotope tracer fluxes—Teorell pointed out that in case of identity of the two ions, the influx is ξ time greater than the outflux, where $\xi = \exp\{V_m/58\}$, with V_m being the membrane potential in mV [37].

Several derivations of the flux-ratio equation for stationary electrodiffusion fluxes have entered the literature; a straightforward derivation that is also valid for an inhomogeneous membrane is given by Sten-Knudsen [38].

7. Active sodium transport exists

In his early studies with frog skin [39], Ussing demonstrated saturation kinetics of the Na^+ influx and observed that the influx is correlated with the transepithelial potential difference (apparently contradicting Ohm's law). During his stay as a Rockefeller Fellow at Bonner's Laboratory at Berkeley, Ussing got the idea that it might be possible to measure the rate of active ion transport as an electric current in a skin bathed with Ringer's of similar composition on the two sides, if the transepithelial potential difference could be eliminated by an external current source [2]. Upon his return to Denmark, he contacted the chemist Karl Zerahn and together they designed the set up. Zerahn wired the circuit and Ussing made the glass chambers and funnels (Fig. 3). They showed that the net flux of Na^+ calculated as the difference between measured unidirectional fluxes, multiplied by the Faraday, is equal to the electric current they had to pass through the skin for bringing the transepithelial electrical potential difference to zero. This current was denoted the 'short circuit current' and the technique is known today as the 'Ussing chamber technique'. The ingenious experimental design allowed them to conclude with no reservations that the electric current carried by sodium ions was driven by free energy derived from cellular metabolism. By exploiting metabolic energy, the sodium ions can be transported in the direction away from the thermodynamic equilibrium distribution. In physiology, the sodium pump was no more a speculative possibility, but a reality [40]. The results were presented as a plenary lecture of the XVIII International Physiological Congress held in Copenhagen in 1950 and published in one of the most influential papers on

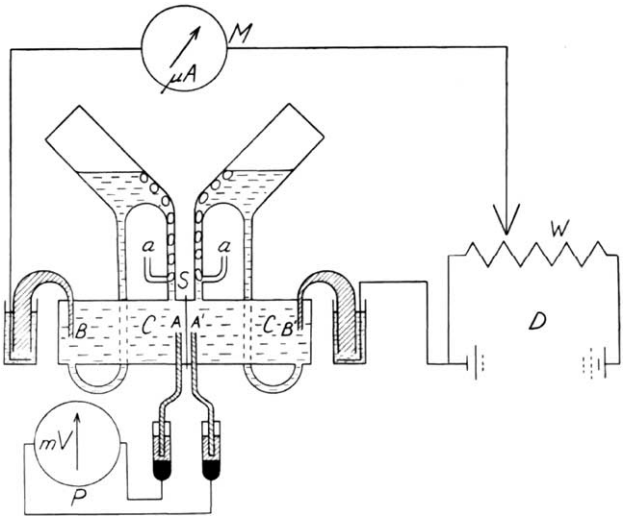


Fig. 3. The short-circuiting technique was introduced for verifying the existence of the sodium pump [40]. The frog skin, S, was clamped between to half chambers, C, containing solutions of identical composition. By bubbling air through the inlets, a, oxygen was supplied to the preparation while pH was kept constant by the CO_2/HCO_3^- buffer. The transepithelial potential difference, which was recorded by P via agar bridges, A, and a pair of matched calomel electrodes, was maintained at 0 mV by passing an electric current through the skin via the Ag/AgCl half cells and agar bridges, B, from an external current source, i.e., a battery, D, in parallel with a manually operated slide rheostat, W. With the radioactive sodium isotope, $^{24}Na^+$, it was shown that the 'short circuit current' is equal to the current carried by sodium ions. It became the method par excellence for studying active transport of ions through planar epithelia. The 'Ussing chamber technique' is used as much today for investigating transport function of both native and cultured epithelia.

epithelial membrane transport [41,42]. Subsequent studies indicated a stoichiometry between the active sodium flux and the component of the oxygen consumption due to active transport of about 18 mole Na^+ pumped for each mole O_2 consumed [43,44].

In 1957, Jens Christian Skou in Aarhus made the all-important discovery that the sodium pump is a membrane-bound ATPase that is activated by Na^+ and K^+. Thus, Skou [45] could conclude that the active sodium transport is fueled by hydrolysis of ATP. As one of the major results of the subsequent studies in Aarhus, Jørgensen [46] succeeded in isolating the kidney Na^+/K^+-pump in pure undenaturated form for investigating turnover numbers, distribution and function of the pump in various sections of the mammalian nephron. In Copenhagen, his group now applies site-directed mutagenesis of the Na,K-ATPase to obtain insights in structure–function relationships at molecular level [47].

With Hilde Levi and Valborg Koefoed-Johnsen, Ussing showed that the ratio of the unidirectional Cl^- fluxes is predicted from the equation for electrodiffusion under open circuit and short circuit conditions with the outer (corneal side) Cl^- concentration varying between 10 and 100 mM [48]. These findings confirmed that under the chosen experimental conditions, Cl^- is not submitted to active transport.

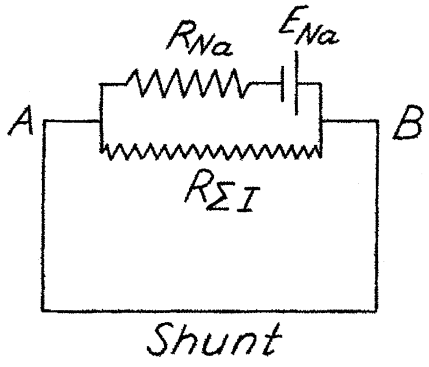


Fig. 4. Ussing and Zerahn represented the frog skin by an equivalent circuit consisting of three elements defined as follows [40], "E_{Na} represents the electromotive force of the Na transport mechanism", "R_{Na} is the resistance to Na movement", and "$R_{\Sigma I}$ is the resistance of the shunt brought about by all passive ions present in the skin". "The lead designated 'Shunt' represents the effect of the applied E.M.F" (that is, the external circuit of Fig. 3). Their studies indicated that the neurohypophysial hormone stimulates the active sodium flux by decreasing R_{Na}.

This is unlike the adrenaline-stimulated preparation that was shown to generate an outward active Cl^- flux, which they correctly interpreted as the result of subepidermal gland activity [49]. One might think that Ussing denied the possibility of active uptake of Cl^- from highly diluted external solutions. This is by no means correct. Ussing was at the Zoophysiological Laboratory when Krogh in 1937 did his experiments with frogs. He felt, though, that the definitive verification of active transport of Cl^- would have to rely also on measurement of the transepithelial electrical potential difference as was done in in vivo experiments in 1954 by Jørgensen, et al. [50] and somewhat later on the skin in vitro [51,52]. Aiming at proving active sodium transport, Ussing [39] deliberately designed his experiment in such a way that putative active transport of other ions, Cl^- as well as H^+, was excluded.

8. The two-membrane hypothesis

In the 1951-paper [40], Ussing and Zerahn propose an electric circuit analogue of frog skin with two parallel branches, which they denoted the 'active' and the 'shunt' pathway, respectively (see Fig. 4). This is the first theory of how a bioelectrical potential is generated by active and passive fluxes of ions. In subsequent permeability studies, Ussing and Koefoed-Johnsen found that the potential difference across preparations with vanishing small anion permeability is the sum of two diffusion potentials, the one for sodium and the other for potassium, respectively (Fig. 5). This limiting condition was approached with either of three protocols, (i) substitution of Cl^- with SO_4^{2-}, (ii) external Cu^{2+} treatment, or (iii) by reducing the external [Cl^-] by a factor of 100, which turned out to shut the 'chloride shunt'. The resulting relationships between the transepithelial electrical potential difference and the external cation concentrations (Fig. 5) were not predicted from the above circuit analogue of frog skin (Fig. 4). Ussing told that he interpreted this contradiction as a paradox, whose elimination required an entirely new way of thinking. This resulted in the two-membrane hypothesis for NaCl transport across an epithelial cell (Fig. 6), which postulates the sodium pump in a K^+ permeable inward facing membrane, while the outward facing membrane is selectively permeable to sodium [53]. In agreement with Glynn's [56] studies of the sodium pump in human red cell leading to the concept of a fixed coupling of Na^+ and K^+ with a stoichiometry of 3/2 [57], Koefoed-Johnsen and Ussing assumed that the sodium pump in the inward facing membrane exchanges cellular Na^+ with internal K^+. With no further assumptions, the model accounted for the measured fluxes, net uptake of NaCl, the skin potential, high intracellular [K^+], low intracellular [Na^+], and the short circuit current. The pump-stoichiometry of $3Na^+/2K^+$ and its rheogenic nature in frog skin were verified

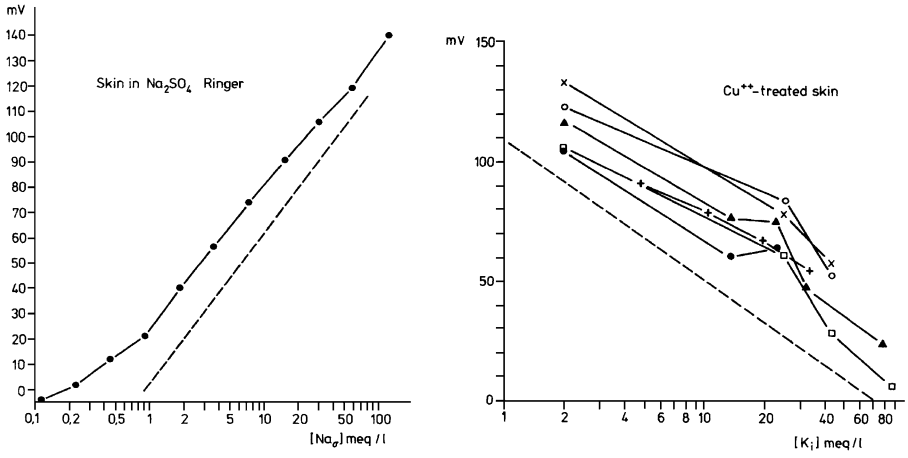


Fig. 5. With 'infinitely large' shunt resistance, under open circuit, the outer surface of the skin behaves like a 'sodium electrode' (left-hand panel) while the inner surface behaves like a 'potassium electrode' (right-hand panel). The theoretical Nernst-slopes are given by dashed lines (monovalent cations of constant intracellular concentration, room temperature). [53].

by Nielsen [58] and Nagel [59], respectively. The intriguing external $[Cl^-]_o$-dependence of the chloride permeability and its regulation by voltage have been investigated in studies that showed the 'chloride shunt' to be localised to a highly specialized minority cell type, the mitochondria-rich cells [60,61].

During the years following the publication of the two-membrane hypothesis, Ussing included studies of the volume of the epithelial cells. They designed a small chamber to be placed on the table of a microscope equipped with water immersion objective, which allowed measurement of the height of the epithelium (Fig. 7). Experiments together with Enid MacRobbie [62] indicated that cell volume is regulated provided chloride ions are present in the inner bath. The membrane processes involved were analysed in detail in Ehrlich ascites tumor cells by Else Hoffmann and her collaborators. Their studies resulted in the concepts of 'regulatory volume increase' and 'regulatory volume decrease' with the first comprehensive review appearing in 1989 [63]. In Ussing's subsequent studies, which were much influenced also by Epstein et al.'s identification of a sodium-gradient driven secondary active Cl^- uptake across the inner membrane [64], it was indicated that similar mechanisms govern volume regulation of frog skin epithelial cells [65]. Independently, a similar conclusion was arrived at in a study on the regulation of the cellular chloride space with $^{36}Cl^-$ as tracer [66]. With this extension, in a logical way [60], the description now also accommodates the puzzling slow ouabain-induced cell volume decrease observed by MacRobbie and Ussing [62], which was difficult to reconcile with the epithelial cell volume simply being determined by the 'pump-leak ratio' as inferred from the first version of the frog skin model [53].

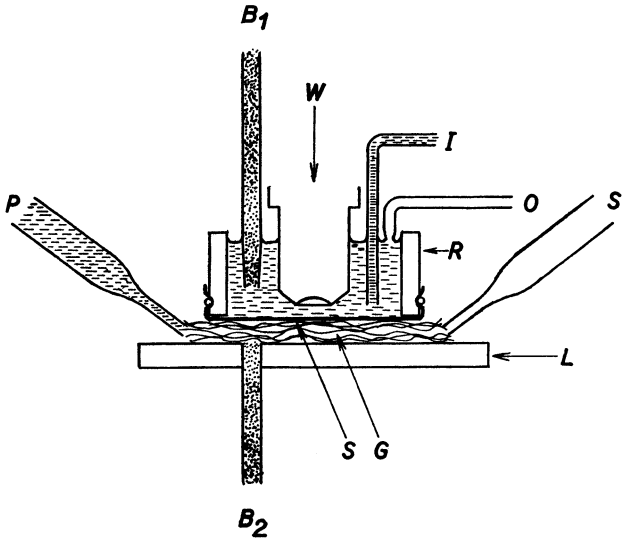


Fig. 7. In their publication from 1961, MacRobbie and Ussing [62] studied the relationships between ion transport, cell volume, and osmotic response of the epithelial cells of frog skin. They designed a cylinder shaped chamber (R) to be placed on a lucite plate (L), which was mounted on the table of an upright microscope with water immersion objective (W). The frog skin (S) was fixed on a pillow of glass wool (G). The transepithelial potential was measured via agar bridges (B_1, B_2), while the two sides of the preparation were continuously superfused with physiological saline solutions (P, S, I, O). They showed that the epithelial cells do not just behave as osmometers, but regulate their volume. Unexpectedly, the cells shrunk when the sodium pump was inhibited by ouabain.

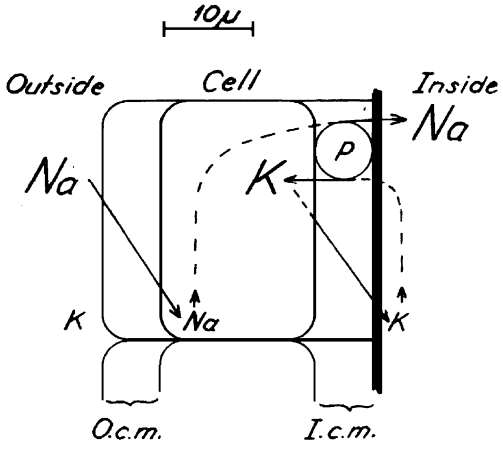


Fig. 6. The two-membrane hypothesis is the first cellular model of a transporting epithelium. O.c.m., outward facing cell membrane; I.c.m., inward facing cell membrane. P with associated arrows, Na^+/K^+ pump and active fluxes. Downward directed arrows symbolise the passive fluxes. The asymmetric distribution of two leak pathways, with the Na^+ leak in the outward facing membrane and K^+ leak in the inward facing membrane, results in active transepithelial transport of Na^+ associated with the building up of a transepithelial electrical potential difference, which makes the Inside relatively positive. The frog skin potential was discovered by DuBois Raymond [54] and further studied by Galeotti [55]. Thus, the title of the Koefoed–Johnsen–Ussing paper, "The nature of the frog skin potential", promises the solution to a problem that was unsolved for more than 100 years. [53].

9. The skin epithelium as a functional syncytium with a paracellular shunt

In Ussing's laboratory, Erich Windhager studied the variation of the intracellular electrical potential with distance from the serosal solution. The impaled cells were localised by dye injection from the recording microelectrode. It turned out that the different cell layers behaved as if they were electrically coupled via intercellular diffusion pathways for Na^+ and other ions [67]. This finding could explain unpublished observations by Koefoed-Johnsen (loc. cit.) that the potassium pool of the whole epithelium exchanges with $^{42}K^+$ added to the inside solution in such a way that the specific activity of K^+ in all layers is about the same at all times during loading with $^{42}K^+$. These data were not easily reconciled with the model of 1958 (Fig. 6), which necessarily assumed the presence of a barrier between the transporting cells in the bottom layer of the epithelium. Therefore, Ussing and Windhager [67] developed methods for more

detailed analyses of the active and the shunt pathway, respectively. They found that exposure of the skin to a hypertonic solution 'apparently increases the permeability of some boundary located at the outer surface of the skin, governing access to the "cellular path" as well as to the "shunt path", although the shunt path is much more affected'. As mentioned above, in its first version, the two-membrane hypothesis depicted the transporting cells identical with the basal cells (stratum germinativum) of the multilayered epithelium. This now had to be abandoned and replaced by a model that assumed the epithelium to behave like a functional syncytium with a shunt between the cells that could be regulated at the level of the outermost living cells [67]. The presence of intercellular junctional passageways throughout the epithelium, and seals (i.e., 'tight junctions') between the outermost cells were independently suggested in a structural study of frog skin epithelium [68,69]. During the same year, Kanno and Loewenstein's intracellular electrical recordings and current injections in *Drosophila* salivary gland epithelium provided the first direct evidence for low resistance membrane junctions between epithelial cells [70].

Frog skin was now a preferred experimental system for studying ion transport in planar high-resistance epithelia. Thus, the revised model of its functional organization challenged several groups to develop and apply new methods for its experimental testing. In a morphological study, Cornelius Voûte showed that stimulation of the active sodium flux across the skin resulted in swelling of the outermost living cell layer [71]. Likewise, in agreement with the new model, with a fast flow technique in Bernd Lindemann's laboratory, it was verified that the sodium selective membrane is located just a few micrometers below the outer border of the epithelium [72], which sodium ions pass by electrodiffusion [73]. In a study using the X-ray microprobe technique, it was verified that perturbation of the membrane transport systems resulted in concerted changes of the concentrations of cations throughout the epithelium [74]. With ^{3}H-labelled ouabain, it was verified that Na^+/K^+ pumps are expressed in all membranes lining the lateral intercellular spaces with no binding sites neither in the apical membrane nor in the membrane facing the serosal solution [75]. Using intracellular recordings with ion-selective microelectrodes [76–78], the selectivity of the two-membranes in series was studied, confirming that in principal cells, K^+ is above and Na^+ below the thermodynamic equilibrium activity. The novel feature of a plasma membrane postulated in the frog skin model [53], i.e., the apical membrane's high resting Na^+ permeability and low K^+ permeability, spurred biophysical studies resulting in the discovery by noise analysis of the amiloride blockable epithelial sodium channel [79]. It has now been cloned and named ENaC [80] (reviewed in Ref. [81]). Likewise, by obtaining power density spectra of blocker-induced noise, epithelial potassium channels were discovered in experiments with frog skin [82]. The compilation of details by studies in many different laboratories added new aspects that were not always concluded to be in agreement with Ussing's basic assumptions. It is now recognized, however, that time-dependent and steady-state relationships between concentrations, membrane potentials and fluxes often turned out to be counter-intuitive and had to await computer-assisted mathematical modelling for rigorous analysis [83].

10. Ussing's frog skin as model for the organization of transporting epithelia

In 1960, Ussing published his studies on frog skin in a comprehensive review of the electrolyte transport literature together with Poul Kruhøffer, Jørn Hess Thaysen and Niels A. Thorn [84]: "The Alkali Metals in Biology". Soon, the monograph was requested and studied by colleagues and students in Europe and USA. The two-membrane hypothesis, the short-circuiting technique, and Ussing's tracer flux concepts dominated the field during the following years and initiated studies of cellular transport functions of other epithelia like gastric mucosa, small intestine and colon, gallbladder, the various sections of vertebrate nephron, airway epithelia, cornea epithelium of the eye, choroid plexus, exocrine glands, etc. The coupling of the passive entrance of sodium to uphill transport of other solutes, driven by the sodium pump, stands as a significant discovery of this period's epithelial membrane studies [85,86]. The two-membrane model of frog skin also stimulated detailed investigations of the cellular sites of action of hormones [87–90] and drugs [91] that regulate the function of these epithelia. The scientific research activity during the decades following the 1960-monograph [84] provides a perfect example of how a new paradigm results in the flourishing of a complex field, where acquired knowledge results also in useful practice [92]. The results of these busy and rewarding years were presented in the four-volume treatise "Membrane Transport in Biology" from 1978, which was edited by Gerhard Giebisch, Dan Tosteson, and Hans Ussing. Ussing wrote the introductory chapter [93] and authored [94] or co-authored [95,96] other chapters. It was now clear that his methods and concepts had been powerful tools for experimental studies also of other epithelia. Furthermore, the two-membrane model with a paracellular shunt, which could be generalized to other transporting epithelia, became the 'definitive framework' of epithelial organization as discussed by Reuss [97].

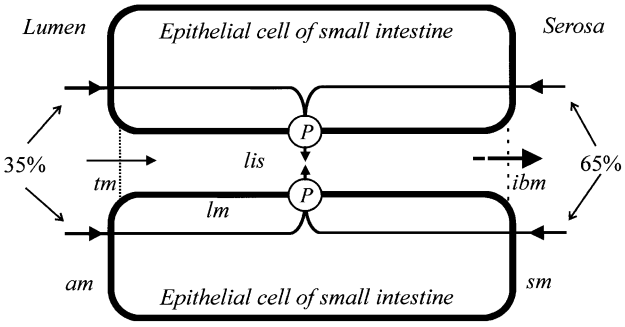
11. Water: pores and solvent drag

In 1952, Ussing returned to the problem about water permeability of cell membranes, which Krogh and Hevesy left unsolved in 1935. His theoretical treatment of water fluxes was based upon the flux-ratio equation, which was derived a few years earlier [27]. In his and Koefoed-John-

sen's experiments with the isolated frog skin [98], the net flux of water was measured as volume flow across the skin, while the unidirectional influx was measured with deuterium water as tracer. Ussing showed that new as well as earlier results were consistent with the assumption that water is transported as bulk flow through pores in the membrane. Using a different approach, Pappenheimer et al. [99] arrived at a similar conclusion regarding fluid transport across the capillary wall. These studies were succeeded by those of Ussing and Andersen [100,101], who investigated how an osmotic water flow influences transport of hydrophilic molecules across the toad skin. The transport mechanism was named 'solvent drag'. The discovery of solvent drag in the skin resulted in demonstration of solvent drag in epithelia specialized for fluid transport [102–104].

Krogh's investigations in the 1930s with heavy water and his discussions with Ussing of common projects with deuterium [10,105–107] led them to realize "in permeability studies of living membranes, to begin with water would be too difficult"[5]. Ussing was confident now that with the pore theory and solvent drag concept, he had a handle on this difficult issue. However, much to his frustration, an inwardly directed net flux of ^{14}C-labeled sucrose, his marker of solvent drag was observed also if the osmotic gradient was driving water across the preparation in the outward direction[6]. Rather than abandoning his base, Ussing introduced the phrase 'anomalous solvent drag' [108,109].

The problems were even worse. Reading of the 1953-paper reveals that Ussing and Koefoed-Johnsen [98] made the quite astonishing discovery that water is transported across the isolated frog skin in absence of an osmotic gradient provided a simultaneous active transport of sodium took place. This result extended earlier in vivo studies [110,111] showing water uptake by frogs from an Na^+-containing Ringer's solution. Ussing and Koefoed-Johnsen [98] suggested that the two fluxes are coupled; wisely, however, they did not propose a mechanism of the putative coupling. The problem was left unsolved and today we can see that in 1953, time was not ripe for a theoretical attack. Investigations in other laboratories, in particular that of Arthur K. Solomon's [112–115], confirmed that fluid is transported through epithelia coupled to active uptake of sodium in the absence of external driving forces for water [116,117]. Surprisingly, it was noticed that the transported fluid is in osmotic equilibrium with the solution bathing the two sides of the epithelium, thus the name isotonic transport. The interest was intensified as it was realized that important body functions depend on this peculiar type of transport, like intestinal fluid absorption, reabsorption of the primary filtrate in kidney proximal tubule, and secretion by exocrine glands. The first piece of the puzzle was contributed by Ussing's own laboratory with his and Windhager's discovery of the paracellular pathway [67], which bridged the gap between high-resistance ('tight') and low-resistance ('leaky') epithelia [118]. This concept and Curran's two-membrane/three-compartment model of the possible linking of a passive water flow to an active solute flux [114] paved the road for the hypothesis that the lateral intercellular space constitutes the coupling compartment [119–121]. In a long series of most elegant and technically demanding experiments pioneered by Kenneth Spring during a period of more than 20 years [122–124], it was shown that the water permeability of epithelial cell membranes often is so large that the osmotic pressure difference required for transporting water through the epithelium is too small for being measured by available techniques. Generally, during the 1980s, the problem was assumed solved (critically reviewed in Ref. [125]): as the result of active sodium transport, there is an osmotic driving force so small that it cannot be measured, only calculated.



am: apical membrane - lm: lateral membrane - sm: serosal membrane
tm: tight junction - ibm: interspace basement membrane
lis: lateral intercellular space - P: Na/K pump

Fig. 8. Sodium recirculation and isotonic transport. By the presteady-state flux-ratio method, transmural unidirectional $^{24}Na^+$ fluxes across toad small intestine were separated into paracellular and transcellular components. Calculations indicated that only about 35% of the sodium ions pumped into the lateral intercellular space (lis) are derived from the mucosal solution via the apical plasma membrane (am). Thus, the recirculation flux, i.e., the flux derived from the serosal solution via the serosal plasma membrane (sm), amounts to ~65% [34]. Model computations showed that isotonic transport requiring this large recirculation flux is generated by a very small concentration difference between lis and the bathing solutions. This surprising result is reconciled with a large diffusion flux across the interface (ibm) between lis and the serosal bath, which exceeds the flux of sodium due to mass flow across ibm. Arrows depict flows of Na^+, but also other diffusible ions (K^+, Cl^-) would have to be recirculated [127]. In small intestine, the major flow of water is paracellular (transjunctional), but the recirculation theory applies as well to epithelia with predominant cellular (translateral) water transport and to epithelia with both translateral and transjunctional flows of water from the mucosal solution into the coupling compartment. No matter which of the two pathways water is following, the recirculation flux decreases and the energetic efficiency of isotonic transport increases with increasing hydraulic permeability between mucosal bath and lis. In the limit of infinite hydraulic conductance, the recirculation flux approaches zero. Thus, Curran's theory of isotonic transport constitutes a limiting case of the recirculation theory.

[5] This is literally how Ussing referred to their discussions.

[6] In Ussing's own words [108]: "And then we did one experiment too many." (loc. cit. page 548).

12. The sodium recirculation theory of isotonic transport

Ussing was impressed by the experimental studies discussed above, but he did not accept the conclusion. In our discussions in Copenhagen, he emphasized that unless it was realized that isotonic transport constitutes a paradox, its mechanism would never be understood. We have to accept, he insisted, that water is passively transported across the epithelium in isotonic proportion with the ions, also under truly transepithelial thermodynamic equilibrium conditions[7]. With this demand, he postulated that the actively transported sodium ions are recirculated. In its present formulation, the theory belongs to the class of theories assuming that the lateral Na^+/K^+-pumps—by making the lateral intercellular space hypertonic—generate an osmotic water uptake from the luminal solution into the lateral space with water being forced from the lateral space into the serosal solution by the associated small hydrostatic pressure difference. As a logical consequence of the fact that the fluid emerging from the lateral space would have to be hypertonic, isotonic transport is not accomplished unless sodium is transported back through the cells for being returned to the lateral space. In 1991, Ussing suggested a protocol based on pre-steady state flux-ratio analysis [29] to be applied to the isolated small intestine for estimating the transcellular- and paracellular components of the two unidirectional sodium fluxes. Later, it turned out that the recirculation flux of sodium could be estimated from these four experimentally obtained fluxes. The experiments that were carried out by Signe Nedergaard [34] confirmed the existence of Na^+ recirculation. During the 1990s, Ussing often turned up in my room for discussing the problems. Especially, we were concerned about the very significant recirculation flux indicated by the experiments (Fig. 8). In order to comprehend the complex interdependence between intraepithelial ion concentrations, driving forces, and the tonicity of the transported fluid, a mathematical compartment model was designed for computer analysis based on classical electrodiffusion–convection theory [126,127]. Our recent computations predict that a large recirculation flux is a necessity for toad small intestine to generate an isotonic transportate. Further to this, we could show that the theory provides logical explanations also for other puzzling and hitherto unexplained experimental findings, including 'anomalous solvent drag'.

Although still controversial, I believe Ussing's ideas and his studies in Copenhagen have provided a promising novel way of analysing solute-coupled water transport. There will be many years of experimental testing ahead of us before we can tell whether the recirculation theory provides a robust framework for studies of the peculiar isotonic transport that has puzzled physiologists for a century. As we have witnessed before, also during these years to come, Ussing's innovative thinking will constitute the intellectual ballast in the discussions of this general feature of epithelia that govern a diversity of important body functions.

[7] Thus formulated, the paradox is evident. Ussing did not care that a small oncotic pressure very likely assists the reabsorption of the ultrafiltrate in vivo. To him, the point was that the isolated proximal tubule, like other leaky epithelia, when exposed to similar solutions on the two sides of the epithelial cells, generates a lumen-to-bath directed transport of isotonic fluid.

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