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Elected ForMemRS 1984

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Hans Ussing was born on 30 December 1911 at Sorø Academy in Denmark, where his father Dr Henrik Ussing was a lecturer and, as historian, a leading Danish folklorist. After his doctoral thesis in marine biology, Hans Ussing came to August Krogh’s laboratory, where he studied protein turnover by using deuterium-labelled amino acids. After World War II, when radioactive isotopes of light elements became available for biological research, Ussing pioneered the development of epithelial physiology by introducing new concepts and theoretical tools, such as unidirectional fluxes, exchange diffusion, the flux-ratio equation, the short-circuiting technique, solvent drag, anomalous solvent drag and the pre-steady-state flux ratio theorem. In studies on frog skin, combining electrophysiology and radioactive tracer technology, he provided the first unambiguous demonstration of active transport of sodium ions. His two-membrane hypothesis of active transport by frog skin initiated studies of epithelial transport at the cellular level in other organs and of the mechanisms of action of hormones and drugs. His discovery of paracellular ion transports bridged the physiology of high-resistance and low-resistance epithelia. With the Na⁺ recirculation theory of isotonic transport he continued his studies of epithelial physiology until shortly before his death. Ussing’s scientific research provided analytical methods and new insights of general applicability for the study of absorbing and secreting epithelia—of equal importance to biology and medicine. Hans Ussing died on 22 December 2000 after a short illness.

CHILDHOOD AND STUDENT TIME

Ussing spent his childhood and youth at the historical Sorø Academy, where his father Henrik Ussing was a teacher at the associated Danish gymnasium (high school). The Academy has quite a remarkable history. The founders of the town of Sorø and the local monastery in the
mid twelfth century belonged to the country’s wealthiest landowning families, which included Absalon, the bishop of Roskilde and founder of Copenhagen. The first Academy was founded by Christian IV in 1623, who erected a building for ball games that has more recently been remodelled to house the Academy’s library. It was copied from a similar building at Hampton Court outside London, where the Danish king’s sister was married to James I. The Academy later housed a large number of Danish poets and writers, including the playwright Ludvig Holberg, who introduced the Age of Enlightenment into Denmark; the poet B. S. Ingemann; the fairytale writer Hans Christian Andersen; and N. F. S. Grundtvig, a famous theologian, church reformer and educationalist. Ussing was born in Molbech’s House, which is one of two beautiful Rococo pavilions built as professorial residences in 1743 during the reign of Frederik V. Later the Ussing family moved to the so-called ‘Minister’s Residence’, whose top floor was occupied by the Minister of Education.

This stimulating cultural setting became the home of the young Hans Ussing, and the breadth of his exposure to the humanities was reinforced by his father, Henrik Ussing Dr phil., who was a historian and a scholar of folklore and toponyms. Henrik Ussing was an assistant lecturer at the Academy when Hans was born, and he advanced to become lecturer in history, Danish literature, and language. He obtained his doctorate for a thesis on the Poetic Elder Edda, which is a collection of poems on mythological and heroic legends from medieval Iceland. Hans’s mother, Jutta Ussing (née Hansen), was a teacher who taught the children to appreciate singing and music. Hans himself learned the violin, played classical music and liked to sing Danish songs with his younger sister. Much later, as a student at the University of Copenhagen, he expanded on these skills by writing songs and sketches for the yearly student shows, and he accompanied himself and other student actors by playing the guitar. The years spent playing and singing in Sorø instilled in Hans Ussing a lifelong love of music.

Even before entering primary school, Hans had already decided to become a scientist. He mentioned that this decision was due in part to the influence of a Danish writer, Carl Ewald, whose short stories and fairytales his father would read to the children at bedtime. Another influence was the beautiful garden and surroundings of his home, and conversations with a good friend of his father, the lecturer Kristen Simonsen, who was an excellent teacher and inspired Hans’s interest in nature. Hans had his own collection of animals and plants, which he used to teach his younger sister. After the family moved to the Minister’s Residence at the Academy he studied physics and chemistry on his own after class. He established a laboratory bench in the cellar of the house, which—his sister recalled—witnessed some very loud chemical experiments. Although his main interests now also included chemistry, he finally decided to study biology. He finished, with summa cum laude, the natural science class of the Academy’s gymnasium in 1929.

In the same summer, at the age of 17 years, Hans Ussing matriculated at the University of Copenhagen in studies of biology and geography. He developed able skills as a field biologist in botany and zoology, but supplemented those studies with physiology and biochemistry and attended lectures in physical chemistry given by Professor J. N. Brønsted. Thus, it was an all-round student who in 1934 was awarded a master’s degree in biology with highest honours, ‘first class with distinction’.
During the summer of 1933, Hans Ussing participated in Lauge Koch’s renowned three-year expedition to East Greenland as a marine biologist and hydrographer. It was his task to collect zooplankton samples and hydrographical data during the summer of 1933, which he analysed along with samples covering all four seasons that had been collected by others during the preceding years. The considerable number of zooplankton organisms obtained from more than 500 hauls constituted the biological material of Ussing’s doctoral thesis entitled ‘The biology of some important plankton animals in the fjords of East Greenland’ (2)*, in which he described the Arctic material and compared it with the zooplankton of boreal waters. For his comprehensive treatise he was conferred the title of Dr phil. in 1938. The two opponents (examiners), one of whom was the Nobel laureate August Krogh ForMemRS, praised Ussing’s description of the vertical distribution of the zooplankton at different seasons of the year, and for revealing how the biological variations relate temporally to variations in the physical conditions of the environment. The thesis was the first scientific description and analysis of an Arctic plankton fauna.

In 1935 Ussing became an assistant scientist at August Krogh’s Zoophysiological Laboratory, which was in the Rockefeller building of the University of Copenhagen, which also housed the departments of Exercise Physiology, Medical Physiology, Biochemistry, and Biophysics. The laboratories received many visitors from abroad on research stays because they were a most attractive place to work for scientists interested in experimental biology. The path leading from marine biology to physiology came from a problem that Ussing had faced in his studies of the Arctic plankton fauna. The larvae of copepods, which constituted a predominant animal group in plankton collections, were not always in a condition that allowed species identification. Ussing recalled (30):

I then got the idea that it might be possible to prepare antibodies against the various larvae and precipitate one species at a time. Before starting on such an enterprise I discussed it with my professor of physiology, August Krogh. He found it attractive but advised me to contact the Serum Institute concerning the procedure. Then he switched to another subject, showing me a small glass vial that contained 1 ml of a clear fluid and asking me what I thought it was. It looks like water, I said. It is water, he answered, but not ordinary water. It is heavy water.

Krogh had asked the discoverer of deuterium, Harold Urey (ForMemRS 1947), for a sample of D₂O, and he was now preparing to study the permeability of living membranes by using heavy water as a tracer for ordinary water. Impressed by Ussing’s resourceful intellect, Krogh invited him to assist in studies on the applications of heavy water in biology. The following five years at the Zoophysiological Laboratory prepared Ussing for his lifelong scientific career in biochemistry and physiology, because there were many exciting challenges ahead for studies of the dynamic state of living cells with isotopes. Besides August Krogh, Ussing also interacted with, and learned from, the protein chemist Professor Kaj Linderstrøm-Lang at the Carlsberg Laboratory and George de Hevesy at the Institute of Theoretical Physics (now the Niels Bohr Institute), who received the Nobel Prize in Chemistry in 1943 for his work on the use of isotopes as tracers in the study of chemical processes. Somewhat later, Ussing was in contact with Brønsted, who worked on theories of energy transformation in chemical reactions.

* Numbers in this form refer to the bibliography at the end of the text.
Krogh taught his students never to approach the laboratory bench without a well-posed working hypothesis. Referring to Krogh, Ussing pointed out that the hypothesis might well be proven wrong, but that even this would be of significance and would lead to progress. Ussing himself insisted on clearly formulated hypotheses developed, as he said, by logical reasoning. In our discussions, he emphasized that by testing an explicit hypothesis, the method for making scientific progress would be deductive, warning against the method of scientific induction and discovery-driven research in experimental biology. According to Ussing, Krogh emphasized that in the discussion of one’s own ideas and hypotheses one is obliged to act as ‘the counsel for the defence’. The ‘judge’, Krogh pointed out, is the fellow colleagues of the scientific community. With his exquisite intellect for grasping the essential design of a complex biological function, in the end Ussing’s ideas were judged to be sound. In the phrasing of Stanley G. Schultz (Schultz & Dubinsky 2001), ‘Ussing had a way of seeing the forest for the trees.’

The opportunities to test new ideas were not restricted by laboratory finances, because Krogh received funds to cover running costs from several granting bodies including the Carlsberg Foundation, the Nordic Insulin Foundation (he chaired the board) and the Rockefeller Foundation in New York. In addition, the laboratory received a substantial income from the sale of equipment that had been designed by Krogh and fabricated by the laboratory’s machine shop. Ussing learned the art of glassblowing and the attitude towards building one’s own equipment from Krogh. Krogh’s laboratory was frequently visited by senior and younger scientists from abroad, who conducted their own projects. Every afternoon they all met for a coffee break in the basement’s animal facility room to discuss problems and progress. At these informal gatherings, Krogh and his younger first assistant, Poul Brandt Rehberg, were the main figures. Ussing recalled how Rehberg, with his keen intellect, would prove that a proposed experiment would be impossible to carry out, while Krogh opposed this view by suggesting an elegant experimental approach that would make the experiment feasible after all. With typical Ussing phrasing, Rehberg would play the character of _advocatus diaboli_ and Krogh that of _advocatus coelestis_. The atmosphere in the laboratory led them to believe that no problem was too difficult if approached with clear analysis and common sense (30).

In 1940 Hans Ussing married Annemarie Fuchs, who accompanied him during the following 53 years as his loving wife, as mother of their two children, Kirsten (1942) and Niels Henrik (1945), and for several years as his secretary. Annemarie always travelled with him and took care of all the important practical matters that he did not care much about. In 1943 Ussing moved on to become a teacher in biochemistry at the Science Faculty of the University of Copenhagen, and in 1945 he was tenured as an Amanuensis at the Zoophysiological Laboratory.

Ussing devoted his life to science, and with a combination of a penetrating intellect, a wide knowledge in natural sciences, confidence in his own judgement, and independence from commonly held ideas he succeeded in founding a new branch of physiology that paved the road to his and other scientists’ great achievements. His analyses of scientific problems and the instruments he developed reflected a clever mind for ingeniously simple solutions. He was very dependent on having someone to talk to, and tried out his ideas on just one colleague at a time. Valborg Koefoed-Johnsen served this role for many years, and other colleagues took over after her retirement. He was never afraid of interrupting our schedule. ‘I don’t disturb you, I suppose’, was his introduction to what might turn out to be an hour-long explanation of a problem that occupied his mind—beginning with so little introduction that it was difficult
to understand him. Ussing was good at listening to his colleagues’ own scientific problems and extremely helpful in clarifying interpretations and suggesting new experiments. Often he turned up in one’s laboratory the following morning with fresh ideas, ready to continue the discussion. He was very encouraging and remained silent when colleagues’ ideas did not stir his interest or seemed badly conceived. Unlike Krogh, in his own writings Ussing avoided criticizing his peers’ scientific papers, nor did he reply to criticisms by fellow scientists, preferring instead to clarify his position in reviews and continue the studies with new approaches. This way of facing scientific controversies was not due to arrogance, but probably reflected an aversion to conflict. After spending some time together with Ussing one learned that he always went a long way to avoid making a person unhappy. These impressions reconcile with colleagues’ experience of Hans Ussing as a board member of granting bodies and research councils. He wanted to consider all talented applicants, even if the sum of money granted to each project ended up being small. Ussing did not enthusiastically favour the democratic ruling of the Danish universities that was introduced in the early 1970s, and he rarely participated in discussions on academic policy with younger colleagues. For these reasons his role as Head of Department was not universally admired even though his general attitude was straightforward and not controversial; he felt the younger generation of university teachers should devote themselves to scientific research, not politics.

**SCIENTIFIC RESEARCH: DIRECTIONS, IDEAS AND IMPACT**

*Body proteins and amino acids*

In 1935, under the leadership of August Krogh, Hevesy, Hofer and Krogh at the Zoophysiological Laboratory used D₂O to measure the permeability of frog skin to water *in vivo* (Hevesy et al. 1935). They showed that the osmotic permeability of ordinary water was about five times the diffusion permeability measured with heavy water. Because a good explanation was not to hand, they decided to abandon the use of heavy water for permeability studies. In his own studies on the toxicity of deuterium-labelled water, Ussing observed that some of the deuterium disappeared from the water and was incorporated into organic compounds, probably proteins. Following up on this observation, Ussing decided to use deuterium-labelled amino acids in exploring the turnover of body proteins.

In discussions with Kaj Linderstrøm-Lang, Ussing developed a method for incorporating deuterium into amino acids by heating with 50% D₂O and 33% H₂SO₄. Deuterium was supposed to be introduced into the C–H position on the Cα atom, and, further, into the ring of aromatic and heterocyclic amino acids. In his first study on one adult rat published in *Nature* in 1938 (1), Ussing showed that liver proteins contained a considerable amount of deuterium after the rat had been fed with deuterium-labelled amino acids for three days, indicating that 10% of the non-labelled amino acids had been replaced by labelled amino acids. The proportion for muscle proteins was smaller, about 2.5%. Next, Ussing developed a novel protocol exploiting the principle that free amino acids of the body fluids take up deuterium in stable C–H positions by transaminations if they are exposed to a constant concentration of heavy water. If the labelled amino acids were then used for protein synthesis, Ussing hypothesized, the deuterium content of tissue proteins should increase exponentially with time. Indeed, by maintaining a near-constant D₂O concentration in the extracellular fluid, the uptake of deuterium by tissue proteins displayed a single-exponential time course from which the
half-time of protein turnover could be estimated (3). This study indicated that the turnover of haemoglobin and myosin is significantly slower than that of liver proteins and other muscle proteins. Shortly after the publication of Ussing’s paper in *Nature*, Schoenheimer et al. (1939) showed that the heavy isotopes of double-labelled dietary D\textsuperscript{15}N-leucine became incorporated into body proteins of rats in body-mass balance. The important general conclusion of these independent studies was that body proteins are constantly synthesized and degraded in such a way that amino acids taken up in food are incorporated into new protein molecules, at the same time as others are catabolized. Of equally great importance, it was clear that the renewal rate of proteins is tissue-dependent and varies with the cellular function of the protein. With his new tool, Ussing had demonstrated how tracer technology could provide fundamentally new opportunities for exploring the dynamic state of living cells.

After the occupation of Norway by Hitler, the Germans took command of Norsk Hydro’s heavy water plant in Rjukan, which became the most heavily defended structure in occupied Europe. As the plant would be vital for the development of an atomic bomb, a sabotage team of the Norwegian resistance army blew it up in 1943. Further biological experiments in Copenhagen with heavy water were now impossible and Ussing was forced to switch to projects on protein metabolism that did not require the use of deuterium-labelled amino acids. With a new method for determining amino nitrogen (amino-N), Ussing showed that the concentration of amino acids in tissues such as muscle, liver and kidney is about the same as that in plasma (4). This was contrary to the dogma at that time, which held that there were 4–10-fold concentration ratios between tissues and plasma. Ussing demonstrated that previous overestimates of amino-N in tissue were due to the high intracellular concentration of glutathione. Taking inspiration from discussions with Rehberg, in his last study with amino acids Ussing joined a growing and most promising field of research. In 1926 Rehberg had invented the creatinine method for measuring the glomerular filtration rate. That pioneering study showed that human kidneys filter about 180 litres of fluid per day—the majority of which is reabsorbed by the tubules, so that the delivered urine amounts to less than 1% of the filtered load (Rehberg 1926). Because the concentration of amino acids in the ultrafiltrate and plasma would be about the same, and because amino acids are normally excreted in only small amounts, it was generally assumed that the amino acids were transported back into blood plasma by tubular reabsorption, either by diffusion because of water reabsorption or by a specific transport mechanism. In Ussing’s investigation of the problem he examined whether the amino acids are handled by amino-acid-selective mechanisms. To perform the studies he then developed new methods for quantifying glycine and a few other amino acids in plasma and urine. His major finding was that the plasma concentration of glycine, and the excretion of glycine by the human kidney, are significantly increased in response to a selectively increased oral intake of glycine. Because the excretion of the other amino acids was maintained constant at the same low level as in fasting individuals, Ussing concluded that the amino acids must be reabsorbed by separate mechanisms along the kidney tubule (5).

The study with radioisotopes of ion transport in biological membranes

**Background**

In 1931 Ernest O. Lawrence in Berkeley invented the cyclotron for accelerating charged particles, for which he earned the Nobel Prize in Physics in 1939. The international stature of Niels Bohr ForMemRS and his Institute for Theoretical Physics with leading physicists around the world made the building of a similar instrument in Copenhagen a priority. Unfortunately,
Hans Henriksen Ussing

the Rockefeller Foundation, which was the obvious philanthropic institution to apply to for economic support, had redirected its emphasis from physics to biology. In 1923 Hevesy introduced the application of isotopes for measuring biological turnover rates of lead by bean plants by using the lead isotope, thorium B (\(^{212}\text{Pb}\)), as the tracer. Like thorium B, all available isotopes at that time were heavy atoms of little interest to biologists, but this situation changed when Frédéric Joliot (ForMemRS 1946) and Irène Curie discovered how to produce unstable isotopes of small elements by bombarding the mother element with charged particles. Not being a biologist himself, Hevesy could not lead the field towards future biological studies with isotopes, so Bohr and Hevesy were both eager to invite August Krogh to collaborate on developing biological applications for isotopes, which the physicists could produce with a cyclotron in Copenhagen and make freely available. At the time, Krogh was focusing on mechanisms of ion uptake by freshwater animals and on ion distributions between cells and blood plasma. It was his hypothesis that a ‘steady state’ is maintained by a balance between passive and active fluxes, which he discussed in a comprehensive review prepared in 1940 but not published until later (Krogh 1946), delayed by World War II. Access to radioisotopes of light, diffusible ions would provide Krogh with new opportunities to test his hypothesis and develop the studies further. The discussions between Bohr, Hevesy and Krogh resulted in an application by Bohr to the Rockefeller Foundation for funds to build a cyclotron in Copenhagen, which was supported by Krogh and Hevesy (Aaserud 1990). The application was approved, and a Danish cyclotron constructed under the guidance of Lawrence’s laboratory began producing artificial radioactive elements in 1938.

Because of the occupation of Denmark by the Nazis, Krogh had to flee to Sweden (Schmidt-Nielsen 1995). He therefore asked Ussing to take over the leadership of projects related to radioisotopes. Ussing accepted, although reluctantly because his own projects in protein biochemistry were developing with satisfying momentum (45). Krogh handed over to Ussing a ‘Memorandum concerning the use of isotopes for determination of ion permeabilities of cell surfaces and living membranes generally’, which was his grant application to the Rockefeller Foundation. To illustrate the different approaches used by Krogh and Ussing when dealing with similar scientific problems, it is interesting to quote from Krogh’s memorandum, which Ussing gave to me a few years before his death (I have omitted sections on protocols and the final section, ‘Technical suggestions’).

Memorandum

concerning the use of isotopes for determinations of ion permeabilities of cell surfaces and living membranes generally.

As a result of studies made mainly during the last 10 years or so and to a considerable extent by means of isotopes it is now recognized

1. that cell surfaces and membranes considered impermeable to certain ions are in fact permeable although the permeability is often of a low order
2. that in view of this permeability the conceptions regarding the mode of preservation of the large differences in ionic composition between cells and their surroundings and between aquatic animals and the water have to be thoroughly revised and
3. that such a revision has in a number of cases demonstrated the existence of an active transport requiring energy of certain ions through cell surfaces and living membranes against a concentration gradient.

When a membrane, say the surface of a red blood corpuscle, is passively permeable to an ion
e.g. potassium, and a many times higher potassium concentration is nevertheless maintained inside
the cell than outside, a quantitative determination of the potassium permeability can not be made by
studying the effect of varying the outside concentration or by any other means which may stimulate
or inhibit the active transport processes, but only by means of a potassium isotope, and even utilizing
the isotope only under certain specified conditions.

We must look upon the cell as being normally in a steady state in which the passive loss of
potassium to the surrounding medium is exactly made good by the active potassium uptake. When
this steady state is scrupulously maintained and the ion in question either outside or within the
cell labelled by addition of a negligible quantity of the isotope the permeability can be measured
[...] and directly compared for different ions, different temperatures, different states of the cells,
provided the condition of steady state is fulfilled.

There are very good reasons to believe that important information on the structure and proper-
ties of cell surfaces and membranes can be attained from such comparisons.

I shall first enumerate a series of problems the solution of which I consider likely to give valu-
able information and next set down some technical suggestions which may be useful in dealing
with these problems.

1. Studies on mammalian erythrocytes.
The erythrocyte membrane is by far the best known among animal cell surfaces. At least in the
rabbit and man it is capable of active ion transport, and reliable figures for ion permeabilities will
be specially valuable.

2. Determinations on muscles.
a. The main work should be done on isolated semitendinosus muscles of the frog which can be
maintained in normal condition for at least 4 days either in the resting condition or performing
contractions at stated intervals. Comparative determinations should be made of the permeability
of the resting muscle at room temp. to Na and K, and of the muscle working at a constant rate.

The effects of different temp., different pH, different Ca concentration could be studied. The limit
of exchangeability for K should be studied by long continued experiments.

b. Similar determinations on the isolated frog heart would probably also give valuable results.
The heart muscle cells contain a fairly large proportion of Na and Cl so that determinations with
radio Na and either Cl or Br might give more clear cut results than those on skeletal muscle.
c. On the muscles of rats kept in K poor diets the relative permeability for Na and K should
be determined—probably in vivo and compared with that of normal rats.

3. The permeability determinations on aquatic animals possessing specific ion transport mech-
nisms present a number of fascinating problems. Only a few will be mentioned.

The gills of Astacus transport only Na, Cl and Br. Those of Eriocheir both K and Na, Cl, Br
and thiocyanate should be tested in solutions approaching fresh water.

One of the interesting points in the location of the permeability is relation to the ion absorbing
surfaces. This could possibly be tested on chironomid larvae on which the absorbing papillae can
be ligated and it is perhaps possible to put the cation mechanism out of action by treatment with
very dilute silver solution.

It should be appreciated that, as was typical for Krogh, his method was comparative when
studying problems of supposedly general nature. For the above set of experiments this
applies to choice of animal species as well as to biological preparations. The principle that
‘for a large number of problems there will be some animal of choice or a few such animals,
on which it can be most conveniently studied’ (Krogh 1929), which is widely but unduly
ascribed to Krogh, actually goes back to Claude Bernard (Bernard 1865). In Krogh’s studies
of physiological mechanisms, be it in humans or in some animal that caught his interest, he often developed the appropriate methods, no matter what level of sophistication was required, rather than choose an organism that would be easier to study experimentally. Examples are provided in the comprehensive biography of August and Marie Krogh by Bodil Schmidt-Nielsen (Schmidt-Nielsen 1995), and in the obituary articles of August Krogh by Archibald V. Hill FRS (Hill 1950) and Poul Brandt Rehberg (Rehberg 1951).

Ussing studied the memorandum, but it did not influence his subsequent choice of project. Whereas Krogh had suggested that the potassium ion had to be actively transported, Ussing chose the sodium ion. He realized that the recirculation of $^{42}$K$^+$ between muscle cells and the interstitial fluid containing a low concentration of potassium ions could not be corrected for during tracer washout experiments, and this would result in an underestimation of the K$^+$ flux across the fibre membrane. He also told me that Krogh did not consider membrane potentials as driving forces for ion flows. Ussing was not confident that a putative active component of the cellular uptake of K$^+$ could be identified, because the large negative intracellular electrical potential might account for the high intracellular potassium concentration. For these reasons he decided to study the transport of sodium ions across the muscle fibre membrane, where there would be only a small chance that the radioactive isotope would re-enter the fibre once it had passed into interstitial fluid with a high Na$^+$ concentration. It was suggested by others that a membrane mechanism actively transports sodium ions from the interior of body cells to the interstitial fluid, which Dean had named ‘the sodium pump’ (Dean 1941). The existence of the pump had not yet been proven experimentally, and among natural scientists the idea of active transport of Na$^+$ was controversial. Ussing liked to recall that when he applied to the Carlsberg Foundation for support for his studies on active transport of Na$^+$, the chemist Niels Bjerrum, who was one of the five directors of the Foundation’s board, invited Ussing to his office to inform him he had been granted the money with the expectation that Ussing would prove the hypothesis wrong.

Frog sartorius muscle: exchange diffusion

Ussing and Levi (8) soaked isolated frog sartorius muscle in a Ringer’s solution containing $^{24}$Na$^+$ until sufficient tissue radioactivity was obtained. Subsequently, $^{24}$Na$^+$ was washed out into Ringer’s solution that was free of the tracer. When plotted on a semilogarithmic scale, the time course of washout showed two straight lines, whose fast component was assumed to be the $^{24}$Na$^+$ accumulated in the spaces between the muscle fibres. The Na$^+$ flux across the plasma membranes was estimated from the slope of the slow component and the measured Na$^+$ concentration of the cell water. The membrane flux thus obtained, which was occurring against a concentration gradient and a potential difference that could be estimated, turned out to be so large that if it were due to active transport it would consume most of the energy production of the muscle fibres. Ussing resolved this paradox by assuming the presence of structural elements in the membrane that permit the exchange of ions in such a way that an equal number of ions having the same charge pass through membrane in opposite directions per unit time. If the affinity of the intracellular and extracellular binding sites is very much higher for Na$^+$ than for K$^+$, exchange of $^{24}$Na$^+$ for $^{23}$Na$^+$ would take place even in the presence of a significant concentration difference across the membrane. For such a 1:1 exchange diffusion, Ussing reasoned, the sum of the thermodynamic work associated with the two equally large but opposite fluxes is zero, thus eliminating the requirement for metabolic energy (7).
Biographical Memoirs

The isolated frog skin

For studies of the putative active transport of sodium ions, the muscle preparation had to be abandoned. Ussing also realized that if the ion activity on either side of the membrane and the membrane potential were not known exactly, it would be impossible to conduct an unequivocal proof of active transport of Na⁺. In the first study applying isotopes, Ussing’s group used ²⁴Na⁺ to investigate whether the uptake of Na⁺ by axolotls is regulated by hormones (6), which would indicate dependence on specific cellular processes. The animals were kept in a 1/50–1/150-fold diluted Ringer’s solution in which ion uptake through the skin matched the sum of Na⁺ excretion through the kidneys and diffusion of Na⁺ through the skin. With the relatively very low external Na⁺ concentration, as a first approximation they could assume that the uptake of Na⁺ by diffusion would constitute a negligible fraction of the total uptake, thus allowing the identification of ²⁴Na⁺ uptake as an active flux. It was shown that the pressor principle from the neurohypophysis stimulates ²⁴Na⁺ uptake with no effect on Na⁺ excretion.

After the ‘failed’ muscle study of 1948, and with the above-mentioned previous success with axolotls, Ussing suggested that the isolated amphibian skin might be a better preparation for studying the active transport of ions. Because the skin of axolotls is difficult to isolate, frog skin became the preparation of choice, which continued to challenge him for more than 50 years. In the first study of the isolated skin, Ussing attempted to measure the flux of Na⁺ that was driven by the active mechanism (9). In a new experimental setup that provided access to both sides of the skin, he developed protocols that showed the interdependence of ²⁴Na⁺ and ⁳⁸Cl⁻ fluxes, and the dependence of these fluxes on the potential difference across the skin. He demonstrated that the Na⁺ influx is close to the total flux of Na⁺ transferred across the skin, which therefore was taken to be an indication of the active Na⁺ flux. In the discussion Ussing assumed that the ‘active transfer’ occurred at the inner membrane of the transporting cells and concluded: ‘the total transport work performed by the transporting cells is greater than indicated by the flux because, due to back diffusion, some Na-ions may be subjected to transport more than once before leaving the skin.’ This early recognition of ‘recirculation’ of molecular species within the epithelium came to occupy his thinking and interpretation of other epithelial functions, which I shall emphasize below. The paper contained also another important result concerning the relationship between the transepithelial potential and the fluxes of ²⁴Na⁺ and ⁳⁸Cl⁻. Ussing wrote: ‘The rather paradoxical situation is that all factors which make the inside solution more positive relative to the outside will increase the influx of the positively charged sodium ions.’ He found that the ⁳⁸Cl⁻ influx was similarly higher as the inside of the skin became more positive, although he did not measure the chloride flux in the opposite direction and left open the question about the mechanism of Cl⁻ transport. The study made it clear that the frog skin presents a scientific problem of its own, namely the causal relationship between the transepithelial ion fluxes and the transepithelial potential. As shown below, Ussing devoted much energy to analysing this phenomenon, which had been discovered by Emil du Bois-Raymond ForMemRS more than 100 years earlier but remained unsolved in 1949.

Unidirectional fluxes and the flux-ratio equation

Ussing’s method in developing the expression of a passive ion flux and its dependence on electrical potential and ion concentration was quite general, considering that it used the Nernst–Planck equation as its starting point. As usual during these years, when faced with difficult problems, Ussing visited Linderstrøm-Lang at the Carlsberg Laboratory. They allowed each other the following weekend for thinking, and then met again to compare their math-
ematical solutions, which seemed to be different (40). To integrate the Nernst–Planck equation through the membrane in the direction of transport they had both assumed constant ion mobility along the path, but whereas Linderstrøm-Lang had assumed a linear concentration profile, Ussing had assumed a linear electric profile.

For a composite membrane, none of the above assumptions was acceptable. Because the radioactive isotope has, for all practical purposes, properties similar to those of the natural non-radioactive isotope, Ussing assumed that it represents the ion it is tracing in such a way that the fraction of the radioactive isotope passing through the skin would be identical to the fraction of the natural isotope that was passing through the skin in the same direction during the same time interval. Thus, introducing the concept of ‘unidirectional fluxes’, the fluxes in opposite directions were denoted as ‘influx’ and ‘outflux’ (later replaced by the term ‘efflux’). By considering the two mathematical equations for stationary net fluxes obtained by Linderstrom-Lang and himself, respectively, Ussing realized that the ratio of the two new variables (influx/outflux) depends only on the ion activity of the bathing solutions and the electrical potential difference between them. This suggested to him that, for electrodiffusion at steady state, the flux ratio of an ion that does not interact with other moving particles is independent of the complexity of the membrane. The expression, which is frequently referred to as the Ussing flux ratio equation, was derived subsequently and published in 1949 (10). Ussing mentioned that his way of discovering the flux-ratio equation for non-homogeneous membranes followed from Brønsted’s teaching of the kinetics of chemical reactions, which is similar to that of transport processes: the logarithm of the flux ratio provides a measure of the affinity of the reaction, and the difference between the fluxes is a measure of the net reaction rate.

The short-circuit technique

For the sodium ion, only a small fraction of the influx across the frog skin could be due to electrodiffusion, illustrating how the flux-ratio analysis helped identify more complicated transport processes. However, Ussing’s argument met criticism because his flux-ratio equation contained activity coefficients for individual ions. During a stay as a Rockefeller Fellow at Bonner’s Laboratory at Berkeley, Ussing became aware of a study by Lund & Stapp (1947) in which they attempted to draw an electric current from frog skin through reversible lead/lead chloride electrodes. By recalculating the currents as ion fluxes, Ussing discovered that they were approximately equal to the Na⁺ fluxes he had measured in Copenhagen. He concluded that it might be possible to measure the rate of active transport of ions as an electric current in a skin bathed with Ringer’s solution of similar composition on the two sides, if the transepithelial potential were eliminated by an external current source (30).

On his return to Denmark, Ussing contacted the chemist Karl Zerahn, and together they designed the setup. Zerahn wired the circuit, and Ussing made the glass chambers and funnels (figure 1). They showed that the net flux of Na⁺ calculated as the difference between measured unidirectional fluxes, multiplied by the Faraday, was equal to the electric current they had to pass through the skin to bring the transepithelial potential to zero. They called the current the ‘short-circuit current’. The study also demonstrated that the net Na⁺ flux and short-circuit current were identical in preparations stimulated by antidiuretic hormone. With this new approach, Ussing concluded with no reservations that the electric current carried by sodium ions is driven by free energy derived from cellular metabolism, and results in a flux in the direction away from thermodynamic equilibrium (11).
With their ‘short-circuit technique’ Ussing and Zerahn had developed a powerful method for identifying and measuring transepithelial active ion fluxes, and provided the first unequivocal proof that sodium ions are actively transported. In the discussion they proposed an electric circuit analogue of frog skin with two parallel branches, which were denoted the ‘active’ and the ‘shunt’ pathway, respectively (see figure 2). The neurohypophyseal hormone stimulated the active Na⁺ flux by decreasing the series resistance of the active Na⁺ pathway. The Na⁺ flux in the active pathway and the parallel flux of ions moving passively by electrodiffusion in the shunt pathway determined the sign and magnitude of the transepithelial potential difference. With this theory of how the frog skin potential is generated by active and passive ion fluxes, Ussing had also obtained an answer to the question about the nature of the frog skin potential that he had raised previously (9). In the electric circuit analogue, the abstraction from the structural organization of the epithelium was carried very far; subsequently this was rectified by the complementary description contained in the ‘two-membrane hypothesis’, as discussed below.

The XVIII International Physiological Congress was to be held in Copenhagen during the summer of 1950; Krogh would have been the president, but he had died in the previous year. Einar Lundsgaard took over the presidency, with Rehberg as general secretary. Krogh had suggested membrane transport as the common theme for three introductory talks, and after Ussing’s return to Copenhagen he was asked to present his studies in a plenary lecture. Alan Hodgkin FRS and Edward Conway FRS had already accepted an invitation to give the other two plenary lectures, so Ussing wanted the scientific results he was to present to be newsworthy.
and of the highest quality. The lecture went off successfully and the paper that appeared in the following year (11) became one of the most influential papers on epithelial membrane biology ever published (Andreoli 1999). In the same year Ussing was promoted to Extraordinary Professor of Zoophysiology, recommended to the Faculty of Science by Rehberg, who became Professor and Head of Zoophysiology in 1945 after Krogh’s retirement.

In a subsequent study, with efflux determined by the long-lived $^{36}\text{Cl}^{-}$ isotope and the net flux by chemical analysis of $\text{Cl}^{-}$, it was shown that the transport of the chlorine ion in frog skin is passive (12). This is unlike the adrenaline-stimulated preparation, which they found to generate an outward active $\text{Cl}^{-}$ flux due to secretion by subepidermal glands (14).

The two-membrane hypothesis

The above circuit analogue of frog skin suggested an interesting experimental approach for analysing the active mechanism. If the ‘chloride shunt’ could be reduced to a very small value, the skin potential should increase and in the limit should be identical to the electromotive force of the ‘sodium ion battery’. This suggested a method for examining the dependence of the electromotive force on the composition of the external solutions. In ion substitution studies Koefoed-Johnsen & Ussing discovered that the potential difference across preparations with vanishingly small anion permeability could be interpreted as the sum of two diffusion potentials, one for sodium ions and the other for potassium ions (19). Whereas most skins exhibited the $\text{K}^{+}$ electrode behaviour on the inside, the apical surface of ‘good skins’ (that is, those with large potentials before ion substitutions) behaved as expected for a $\text{Na}^{+}$ concentration battery. These relationships were not contained in the above circuit analogue of frog skin (figure 2), which ascribes to the active transport of $\text{Na}^{+}$ the sole responsibility for the skin potential. Therefore the set of new of observations required quite a different consideration, which one would think might have been in their minds when planning the above experiments (figure 3). However, nothing was noted about this and the conflicting descriptions were a ‘riddle’ for Ussing (40), which could be solved by adding to his ‘transporting cell with an active mechanism in the inward facing membrane’ (9) two additional properties, namely a $\text{K}^{+}$-selective inward-facing membrane and a $\text{Na}^{+}$-selective outward-facing membrane (figure 4). The ‘two-membrane hypothesis’ accounted for the uptake of $\text{NaCl}$, the skin potential, the high intracellular $\text{K}^{+}$ concentration, the low intracellular $\text{Na}^{+}$ concentration, and the short-circuit current. In complementing the circuit analogue proposed in 1951, the advantage of the new
description was that it related the anatomical structure of the epithelium to its function, which expanded the studies in quite new directions.

Ussing’s laboratory had now moved to another address, at the university’s botanical garden, where he became Professor of Biochemistry and Head of the Institute of Biological Chemistry. The first project was to develop a method for testing the new model that would be independent of potential measurements, by taking advantage of predicted changes in cell volume in response to alterations in the composition of the bathing solutions. Ussing designed a chamber that could be placed on the stage of a microscope and viewed with a water-immersion objective, which allowed the height of the epithelium to be recorded with an accuracy of ±1 μm, providing a measure of epithelial cell volume (figure 5). Experiments with Enid MacRobbie (FRS 1991) (21) confirmed the relatively large K⁺ permeability of the inward-facing membrane, and that the product of the cell concentrations of K⁺ and Cl⁻ is larger than that of the solution bathing the inside of the skin, which was attributed to the activity of the Na⁺/K⁺ pump in the inward-facing membrane. Similarly, the impermeability of the membranes to the sulphate
ion was verified, and the intracellular Cl$^-$ concentration was estimated to be 40–50 mM under normal conditions, on the basis of the change in epithelial volume after substitution of Cl$^-$ for SO$_4^{2-}$. It was shown that antidiuretic hormone stimulates osmotic water uptake by increasing an otherwise negligible water permeability of the outward-facing membrane. These results, which were easily reconciled with the two-membrane hypothesis, were all new and demonstrated how powerful a method Ussing had invented. However, the study also raised two problems that were not solved until about 20 years later. Unlike the simple osmotic responses in sulphate Ringer, on dilution of the chloride Ringer on the inside of the epithelium, acute swelling was followed by a return of the cell volume toward the original value. When ordinary Ringer was again added on the inside, the epithelium shrank initially, as expected, but
then swelled back to the original volume with ordinary Ringer. These complicated responses were not predicted by the model, and it was also troublesome that the cell swelling expected after inhibition of the pump with ouabain was not confirmed, although this finding was not discussed explicitly. Either the volume stayed the same during ouabain exposure or, worse, it decreased! In further experiments Ussing searched for expansions of the model that could accommodate the conflicting observations. Both problems found their logical explanation once he realized that the cellular Cl\(^-\) pool, which determines the epithelial cell volume, is maintained above thermodynamic equilibrium by regulated basolateral Na\(^+\)-gradient-driven co-transport of Na\(^+\), K\(^+\) and Cl\(^-\) in parallel with regulated Cl\(^-\) and K\(^+\) permeabilities (32, 34).

In the planning of these studies, Ussing took inspiration from the hypotheses of his colleague Else Hoffmann and her co-workers about how cells of the body regulate their water volume in anisosmotic environments (see Hoffmann et al. 2009).

The revised frog skin model: intraepithelial syncytium and paracellular shunt

Ussing and Andersen (17, 26) observed that the resistance decreases markedly when glucose, urea and certain other substances are added to produce hyperosmolar concentrations outside the toad skin. In further studies with frog skin, Ussing found that the decrease in resistance is caused by increased permeability to both anions and cations, with little change in the short-circuit current. He therefore hypothesized that the introduced leak is located between the Na\(^+\)-transporting cells. As visiting scientist at the Institute of Biological Chemistry, Erich Windhager performed microelectrode studies on renal tubules. Ussing therefore asked Windhager to help test his hypothesis by recording intracellular potentials in the frog skin. The setup was modified so Windhager could measure the intracellular potential in different cells of the multilayered epithelium and identify the impaled cell by injecting dye from the recording electrode. It turned out that the different cell layers behaved as though they were electrically coupled to one another through intercellular diffusion pathways for Na\(^+\) and other ions. In the revised frog skin model, Ussing and Windhager depicted the epithelium as a functional syncytium with a paracellular shunt that was regulated at the tight junctions in the outermost layer of living cells (22). The study made it clear, but did not discuss, that Windhager’s measurements revealed a new problem. The assumption of a high Cl\(^-\) permeability of the inner membrane with an intracellular Cl\(^-\) concentration of 40–50 mM was not compatible with the large negative membrane potential of about \(-70\) mV. As mentioned above, the solution to this problem had to wait until Ussing clarified that the inner membrane has low Cl\(^-\) conductance and has mechanisms that maintain the intracellular Cl\(^-\) concentration above thermodynamic equilibrium (32, 34), in agreement with contemporary studies in other laboratories (Ferreira & Ferreira 1981).

The new model implied that the Na\(^+\)-selective membrane is essentially the outward-facing membrane of cells immediately beneath the cornified layer of dead cells. This was tested, in collaboration with Cornelis Voûte, with a method allowing simultaneous morphological and electrical studies in skins from frogs that were in different physiological states. In agreement with prediction, they observed a marked and reversible swelling of the outermost living cell layer in response to stimulation of the Na\(^+\) current by voltage clamping (24).

During the collaboration with Voûte in 1971, Ussing and his colleagues moved to the new ‘August Krogh Institute’ together with the Zoophysiology Laboratories and the Laboratory of Exercise Physiology at the Rockefeller building.
The flux-ratio equation under non-stationary conditions

Ussing reasoned (27, 28) that, for a single pathway of a multi-membrane system under steady-state conditions, the ratio of the unidirectional tracer fluxes would remain constant from the moment that the tracer first appeared on the trans side of the membrane and that it would be equal to the flux ratio under steady-state conditions, regardless of the transport mechanisms involved. This surprising result was unbelievable to many, including Ove Sten-Knudsen, who nevertheless proved Ussing correct in a demanding mathematical treatment of the problem (31). A corollary to the theorem is that if the ion makes use of more than a single pathway with different passage times and flux ratios, the ratio of the unidirectional fluxes should be time-variant. This result is of no use for studies of single plasma membranes, for which steady-state fluxes are achieved within fractions of a second. However, it is interesting for studies of epithelia with large cellular ion pools, in which isotope fluxes require minutes or hours to approach the steady state.

As emeritus professor from 1982, Ussing demonstrated the operational significance of the above remarkable theoretical result by developing a method for separating the steady-state fluxes through the cellular (slow) and paracellular (fast) pathways without the need for inhibitors, ion substitutions, voltage clamping, or any other perturbations of the physiological state of the epithelium (33). The method was used to analyse transepithelial Na⁺ fluxes in frog skin. By assuming that the oppositely directed transcellular fluxes pass through the Na⁺/K⁺ pump and that Na⁺ is not subjected to single-file diffusion along the pathway, Eskesen and Ussing estimated the electromotive force of the pump under different physiological conditions (35). They found that the electromotive force approached 200 mV as the net flux through the pump approached zero, in agreement with a phosphorylation potential of ATP of about −60 kJ mol⁻¹.

It was suggested that the smaller values of the electromotive force associated with large active Na⁺ fluxes are caused by a decrease in the cytosolic ATP/ADP ratio near the pumps, and it was reasoned that the diffusion of ATP from mitochondria to pumps in the lateral membranes should be included in the series resistance of the circuit analogue developed previously (11). In a subsequent paper (36) they provided evidence for single-file diffusion through K⁺ channels in frog skin with a K⁺-permeable outer membrane by taking advantage of another implication of the non-steady-state flux-ratio theorem. Ions redistribute so slowly during ⁴²K⁺ fluxes across the syncytium that steady-state fluxes are not achieved within a reasonable time. However, the ratio of the steady-state unidirectional K⁺ fluxes can be obtained from pre-steady-state unidirectional ⁴²K⁺ fluxes. The flux-ratio exponent that approximates the number of ions in the K⁺ channel pore during single-file diffusion was found to be different from unity with an average number of about 2.5 (range 1.77–3.14). With the rate-limiting barrier being the outward-facing membrane and the pump activity significantly decreased by the omission of Na⁺ from the outer bath it was concluded that single filing takes place in the K⁺ channels of the apical membrane. Once more, Ussing had invented a most powerful method for revealing details of ion transport processes across living membranes of considerable anatomical complexity. I shall return to this issue in the section below on Na⁺ recirculation in leaky epithelia performing isotonic transport.

Epithelial water transport

Ussing’s investigations of water transport, although having clear connections to his ion transport studies, constitute a parallel line of research that offered many difficult challenges. The approaches displayed great originality and produced many surprising findings on interactions
between transepithelial ion fluxes and water fluxes. Converging on the general physiological problem of isotonic transport and his Na⁺ recirculation theory, Ussing finished his contributions to membrane biology with a novel conception of the regulation of solute–water interactions in epithelia. This was accomplished by applying methods and theoretical tools that he himself had developed over a period of 40 years, namely the short-circuit technique, the double-labelling isotope tracer method, the non-stationary flux-ratio method for separating cellular and paracellular cation fluxes, and the concept of solvent drag as revealed by flux-ratio analysis of paracellular fluxes.

Water permeability and water-filled pores

According to Ussing (13) the essential problem raised in Hevesy et al. (1935) was that the ratio between influx and efflux of water could not be equal to the ratio of outside and inside activities of water. He considered the possibility that the water molecules, superimposed on their rate of diffusion, may show the rate at which the solvent flows through pores of the membrane. Alternatively, the water transport might be active. Ussing and Koefoed-Johnsen (15) addressed these questions using the flux-ratio equation of H₂O fluxes as a theoretical tool. The net flux of water was measured as volume transfer, whereas the unidirectional influx was estimated with heavy water as tracer. The study with isolated preparations confirmed the findings of Hevesy et al. (1935) with frogs in vivo. The permeability to D₂O was not changed much by antidiuretic hormone, whereas the bulk water flow more than doubled. They concluded that both new and earlier results were consistent with the hypothesis that water passes through pores as bulk flow, and suggested that the hormone opened up pores or created new pores with little effect on the total area available for diffusion of water. This paper provided the first evidence for pore-mediated water transport in epithelia, and together with the paper by Pappenheimer et al. (1951) on fluid transport across the capillary wall it established new directions for the study of the water permeability of biological membranes. Dainty & House (1966) pointed out that the discrepancy between osmotic and diffusional permeability might be attributed to an unstirred layer on the outside of the skin. Ussing acknowledged the criticism that the stagnant layer influences the osmotic permeability to a smaller extent than it does the diffusion permeability (29). Nevertheless, the diffusion permeabilities obtained (15) were of similar magnitude to those obtained in Dainty & House (1966) with rapid stirring, and subsequent studies have confirmed that antidiuretic hormone ‘creates’ new water channels in the apical membrane.

Solvent drag

The study with Bernhard Andersen (18) tested the pore hypothesis by considering that water flow through pores would speed up the flux in one direction of any molecule that could permeate the pore, and slow down its flux in the opposite direction. Two test substances of low lipid solubility were selected: thiourea and acetamide. Both were synthesized in two labelled forms: thiourea was labelled with ³⁵S and ¹⁴C, whereas acetamide was tagged with ¹⁴C in the carbonyl or the methyl group. Net water flux was measured volumetrically, and influx was measured with D₂O. As expected, the flux ratio (influx/efflux) of both test substances was increased after stimulation of osmotic water uptake by antidiuretic hormone; however, the study also reported another important finding. A plot of the logarithm of the ratio between the rate coefficients of the two test compounds against net water flow showed scatter about the same straight line going through the origin. Thus, none of the compounds were subjected to
active transport, and the pores through which the two compounds were transported seemed uniform with respect to dimensions. It was concluded that water flowing from the outside to the inside of the skin speeded up the inward flux of the two test substances. This transport mechanism Ussing named ‘solvent drag’.

At the beginning, solvent drag was associated with pores in the plasma membranes of the epithelial cells. After the extension of the two-membrane model (22) it became equally likely that the ‘drag’ is exerted in narrow pores within the tight junctions between adjacent cells. Today the latter interpretation is preferred because both reconstituted and cloned water channels of cell membranes (aquaporins (Preston et al. 1992)), generally, are permeable only to water.

‘And then we did one experiment too many’

Ussing told me that Krogh’s investigations with heavy water (Hevesy et al. 1935) led them to conclude that, in permeability studies of living membranes, to begin with water would be too difficult. This was emphasized once more in a 1955 study with Kalman (16) showing uptake of water through toad skin with Ringer on the outside, which could be stimulated by antidiuretic hormone if the outside solution was isotonic NaCl but not if it was isotonic sucrose. With the driving force for transepithelial water flow being zero, these observations were not easily explained by any ideas of the time.

However, with experimental evidence for water uptake through pores and the demonstration of solvent drag, at least the problems arising from Hevesy et al. (1935) seemed solved. These results were important for Ussing’s thinking because they indicated coupling between fluxes of different molecular species beyond the coupling between Na\(^+\) and Cl\(^-\) that results from the skin potential, as shown in his previous studies (9, 11, 12). To investigate this new area of solute–solvent interactions, Ussing attempted to verify hormone-stimulated coupled fluxes between water and organic test substances in the presence of a reversed osmotic gradient. Under these conditions, an outwardly directed driving force for water transport was established by placing hypertonic solutions on the outside of the skin and Ringers on the inside. With this protocol, the skin conductance is dominated by a non-selective shunt (22). Ussing speculated that the active Na\(^+\) flux would no longer be identical with the short-circuit current if a fraction of the sodium ions that were pumped into the lateral space could return to the outside solution through leaky junctions.

Paper (23) dealt with the above problem by combining the measurement of oxygen consumption with radioactive tracer fluxes of Na\(^+\), K\(^+\), SO\(_4\)\(^{2-}\) and sucrose in skins that had been made leaky by exposure of the outside to a hypertonic solution by adding urea to a concentration of 200 mM (figure 6). The first series of experiments demonstrated the increased metabolic cost of the short-circuit current, which would be in agreement with Na\(^+\) recycling. However, a further test of this hypothesis, by measuring unidirectional Na\(^+\) and SO\(_4\)\(^{2-}\) fluxes, showed that the net flux of Na\(^+\) was larger than the short-circuit current and that the inward flux of SO\(_4\)\(^{2-}\) was significantly larger than the efflux of SO\(_4\)\(^{2-}\) at transepithelial electrochemical equilibrium. All of these results were unexpected, and were not considered to bring meaningful insights about the shunt in the leaky epithelium. Rather than stopping further experimentation, Ussing decided to try ‘a fresh attempt to demonstrate the hypothetical intercellular shunt’ (Ussing’s phrasing) by measuring the sucrose efflux before, during and after exposing the epithelial side of the skin to the hypertonic solution. As predicted, sucrose efflux increased reversibly during hypertonic treatment. According to Ussing (23), ‘And then we did one experiment too
many.’ That is, with a similar protocol during the period with outwardly directed water flow, the influx of sucrose increased reversibly over and above the efflux. In other words, the net flux of sucrose supposed to be driven by the flow of water was opposite to the direction of water flow. For this phenomenon, Ussing introduced the concept of ‘anomalous solvent drag’.

In retrospect, Ussing stated: ‘We got the surprise of our lives’ (26).

Ussing showed that the net flux of sucrose is linearly dependent on the short-circuit current (23), suggesting the possibility that anomalous solvent drag is fuelled by ATP hydrolysis at the Na⁺/K⁺ pumps. However, he did not a priori exclude other mechanisms, and when it turned out that asymmetric sucrose fluxes were observed in the complete absence of sodium ions, for example with K₂SO₄ Ringer on both sides (23), other ways of thinking about anomalous solvent drag were necessary. This forced him to realize that the asymmetric urea concentration itself represents a potential energy source, which could induce asymmetric solute fluxes across the skin resembling active transport but mediated by a circulating water flow within the epithelium. To explain how momentum could be transferred from the driving species to
the driven radiolabelled species by solvent recirculation, Ussing illustrated his idea by an epithelial compartment model in which the lateral intercellular space was an intraepithelial osmotic coupling compartment (26). Diffusion of the driving species (urea) into the lateral intercellular spaces from the outside through the leaky junctions would make the lateral spaces hypertonic. Osmotic water uptake from the inside bath via the cells into the lateral space would then recirculate back into the inner solution as a result of the very low reflection coefficient of the basement membrane. The driven species—the small radioactive ions and organic molecules such as $^{36}$Cl$^{-}$, $^{35}$SO$_4^{2-}$, $^{14}$C-urea or $^{14}$C-mannitol, which enter the lateral space from the outside through the leaky tight junctions—would be speeded up relative to their diffusion by this water flow from the lateral spaces to the inner solution, whereas radioactive molecules of the same species entering the lateral space from the inside would be impeded in their diffusion. Therefore the flux ratio (influx/efflux) of the driven species would be greater than unity under conditions of equal electrochemical activity on the two sides of the skin, resembling active transport. However, unlike conventional solvent drag, the solute–solvent interaction by recirculating water would take place in narrow spaces between the cells and in pores of the basement membrane. The hypothesis was particularly satisfying because it readily explained why the driving species could be a wide spectrum of chemical compounds, such as urea, creatinine, dimethylsulphoxide or NaCl, and that the driven species similarly were unrelated to chemical structure, as exemplified by sucrose, galactose, mannitol, urea, chloride or sulphate. Inulin was not subjected to anomalous solvent drag, indicating that the driven species would have to be below a certain size for permeating the leaky junctions. The limiting size turned out to be that of raffinose. Ussing and Johansen (25) showed that the flux ratio of the driven species decreases if the solution bathing the inside of the leaky skin is made hyperbaric.

**Solute-coupled water flow and isotonic transport: the Na$^+$ recirculation theory**

In certain types of secretory and absorbing epithelia, water and ions are transported in isotonic proportions in the absence of a transepithelial driving force for water. To Ussing this was a fascinating paradox, which he began to study in 1985. At that time, Ussing and I spent the autumn and winter months together at Kenneth Spring’s laboratory at the National Institutes of Health (NIH) in Bethesda, USA. With Spring’s method of measuring cell volume changes by video imaging of an intact epithelium, the three of us together with Kevin Foskett studied the transport of Cl$^-$, H$_2$O and Na$^+$ by mitochondria-rich cells of amphibian skin (37–39). One morning Ussing entered the laboratory to tell me that he had analysed Karen Eskesen’s unpublished flux data. With the epidermal ion fluxes blocked, their study aimed at separating cellular and paracellular fluxes of Na$^+$ and K$^+$ in the subepidermal glands by the non-stationary flux ratio method on the basis of the hypothesis that water passes through the glandular epithelium of frog skin between the cells. One way of testing the hypothesis was to see whether the paracellular Na$^+$ flux was being subjected to solvent drag. To Ussing’s surprise, his calculations indicated that a fraction of the sodium ions that had entered the luminal space through the tight junctions was taken up by acinar cells and pumped back into the serosal side by the basolateral Na$^+/K^+$ pumps. He was quite animated by the result and asked me to postpone the experiments to discuss a new idea with me. We spent most of the day working on the problem, during which he explained how the recirculation of ions energized by the Na$^+/K^+$ pumps might be crucial to achieve a truly isotonic secretion by the acinar epithelium. This was a completely incidental recognition prompted by measured Na$^+$ and K$^+$ fluxes that he could not otherwise make sense of; these are explained in more detail below.
After that, the problem of isotonic transport occupied Ussing’s mind constantly. His thinking about it was quite unusual (44), resulting in mathematical treatments published in papers with Eskesen (41) and Kristensen (43). Ussing’s approach to solute–solute interactions as exemplified in these two papers prevented enthusiastic support for his ideas, and Ove Sten-Knudsen, with whom Ussing tried to discuss the problem, did not hide his scepticism. Whereas Ussing maintained that isotonic transport results from fluxes along both cellular and paracellular pathways, Sten-Knudsen at that time was more inclined to support the idea that isotonic transport is the result of molecular coupling of water and sodium ions within a single transport protein. Sadly, this disagreement prevented them from further discussion of a major unsolved problem in epithelial physiology. With reference to our discussions at the NIH mentioned above, it is obvious that Ussing’s formalistic treatment (43) was a way to rationalize a general solution to isotonic transport, which he had conceived intuitively. In his theoretical considerations he aimed at the inherent physical problem of solute-coupled water transport and a way to overcome it for achieving isotonic transport. Ussing was therefore in need of a mathematical treatment, and this he had to develop himself before presenting the experimental data (41).

The logical experimental strategy was to compare the paracellular fluxes of Na$^+$ with those of K$^+$. Glandular secretion was stimulated by noradrenaline, and ion fluxes through the epithelial cells were blocked or reduced by inhibitors. At transepithelial thermodynamic equilibrium the flux ratio of both cations was found to be significantly above unity (efflux/influx), with the flux ratio for K$^+$ being larger than that for Na$^+$. These findings provided the experimental evidence for paracellular solvent drag on the two cations. Because the diffusion coefficient of Na$^+$ in water is smaller than that of K$^+$, solvent drag would make the flux ratio of Na$^+$ larger than that of K$^+$, contrary to observation, which led Ussing to conclude that Na$^+$ was being recirculated. It was pointed out that, if properly regulated, such a reuptake of Na$^+$ by the acinar cells would be necessary for achieving an isotonic primary secretion (41). Hoffmann and Ussing (42) discussed the possible significance of epithelial cell volume changes as a signal for regulating the activity of ion channels and the basolateral Na$^+$,K$^+$,2Cl$^-$ co-transporter in maintaining an isotonic secretion by the acinar epithelium.

Ussing and Eskesen (41) did not discuss the assumption of a purely passive transport of K$^+$. However, if there were active K$^+$ secretion the Na$^+$-recirculation flux would have been overestimated—or perhaps non-existent. This forced Ussing to look for another cation to trace paracellular fluxes and to test the assumption of passive K$^+$ transport (46). In an ingeniously conceived inspiration, the choice fell on the caesium ion. In cleverly designed experiments with frog skin, Ussing (47) showed that $^{134}$Cs$^+$ is taken up by the Na$^+$/K$^+$ pumps, becoming irreversibly trapped by the epithelial cells. By using carrier-free $^{134}$Cs$^+$, K$^+$ channel inhibition by caesium ions was reduced to a minimal level, so Ussing could conclude that once Cs$^+$ had been taken up into the epithelial cells by pumps, the cation selectivity of the K$^+$ channels prevented Cs$^+$ from returning to the bathing solutions. The significance of this surprising experimental result was that a transepithelial flux of $^{134}$Cs$^+$ had passed the epithelium exclusively between the cells. With the $^{134}$Cs$^+$ method for tracing paracellular fluxes of alkali-metal ions, in a new series of experiments on frog skin glands Ussing verified active K$^+$ secretion. Nevertheless, Na$^+$ recirculation could be demonstrated with this method as well (46). In a subsequent study at our institute confirming active K$^+$ secretion, we identified the luminal K$^+$ channel of the acinar cells by the patch clamp technique (Sørensen et al. 2001). Similarly, a small luminal Na$^+$ channel of frog gland acinar cells was discovered that was activated in response to hormone-stimulated secretion (Sørensen et al. 1998; Sørensen & Larsen 1999).
Ussing reasoned that Na⁺ recirculation was necessary for isotonic transport generally, and with Signe Nedergaard he tested the idea on the absorbing small intestine of the toad (47). The recirculation flux was estimated by a quite different method, which was independent of measurements of paracellular ¹³⁴Cs⁺ fluxes. Unidirectional Na⁺ fluxes through the cellular and the paracellular pathway were separated by the non-stationary flux ratio method. In the analysis of the flux pathways he took advantage of the finding that the Na⁺/K⁺ pumps are localized in the lateral membranes of the epithelial cells, which express Na⁺,K⁺,2Cl⁻ co-transporters in the basolateral membrane. However, if Na⁺ recirculation were involved, the Na⁺ recirculation pathway and the Na⁺ pumps, as in the glandular epithelium, would have to be localized to different membrane domains. Thus, Ussing assumed that the co-transporter is localized in the basal membrane of the cells. (The type of entrance pathway for Na⁺ in the luminal membrane of absorbing epithelia is of no importance for the proposed mechanism of isotonic transport. It might be a Na⁺/H⁺ exchanger, a Na⁺,K⁺,2Cl⁻ co-transporter, a Na⁺-coupled uptake of glucose and amino acids, or a Na⁺ channel.) With this assumption and his previously derived theoretical result (13) that a source (the Na⁺/K⁺ pump) delivering a tracer flux into a reversible pathway (the lateral space) would not influence the ratio of unidirectional fluxes through the pathway, Ussing developed a novel set of equations for estimating the Na⁺ recirculation flux, which turned out to be substantial (47).

Ussing’s last paper was an invited review on the putative role of paracellular pathways for isotonic transport (48). We introduced another approach for elucidating the fundamental problem of isotonic transport. Because of diffusion superimposed on the convection of solutes, the fluid flowing out of the hyperosmotic and hyperbaric lateral intercellular space would be hyperosmotic relative to the fluid in the lateral space and to the surrounding solutions of similar composition. Thus, recirculation of ions would be a way of regulating the composition of the transported fluid to the desired tonicity. According to the new treatment, the magnitude of the Na⁺ recirculation flux depends on the water permeability of the apical barriers separating the lateral space from the luminal solution (48) (Larsen et al. 2000, 2002). With the very low water permeability of small intestine, a large recirculation flux was to be expected, in agreement with the previous experimental estimate (47).

The applicability of Ussing’s frog skin studies

In 1960 Ussing wrote a comprehensive monograph on electrolyte transport across biological membranes, including methods, observations and interpretations of his own work on frog skin. It was published as part I (The alkali metal ions in isolated systems and tissues) of the book The alkali metals in biology (20), and Poul Kruhøffer, Jørn Hess Thaysen and Niels A. Thorn wrote the various sections of part II, comprising literature on the alkali metal ions in the organism. In his opening remarks as chairman for a CIBA Foundation symposium in 1960, Alan Hodgkin wrote (Hodgkin 1960):

Most of you will have seen the magnificent book by Professor Ussing and his colleagues on The alkali metal ions in biology. … Nowadays the demands of conferences, scientific research and life in general are such that people rather rarely write a full book about their own subject. This is a pity, and we all owe a great debt to Professor Ussing and his colleagues for producing this excellent work.

Ussing’s contributions placed the original frog skin studies performed in Copenhagen on equal terms with work on nerve, muscle and red cells as an experimental model system in membrane transport. By founding a new experimental tradition, Ussing’s theoretical and
experimental tools influenced numerous studies on the transport physiology of other epithelia such as the gastrointestinal tract, the gallbladder, kidney tubules, airway epithelia, the cornea epithelium of the eye, the choroid plexus and exocrine glands. Today the ‘Ussing chamber technique’ is indispensable in studies of epithelial physiology, whether it be in vitro preparations, confluent tissue cultures with diseased genes, or cultures expressing engineered proteins. By applying Ussing’s theoretical results and experimental approaches, highly fruitful work on other types of biological membranes has studied new transport mechanisms such as the Na⁺-coupled uptake of sugars and amino acids, secondary active transport, and exchange diffusion between non-identical ion pairs (Tosteson 1959; Wieth 1979; Aronson et al. 2009).

The two-membrane hypothesis introduced and clarified problems in the fields of biochemical regulation of epithelial function, epithelial differentiation, and the pharmacological and biophysical identification of individual ion transporters (Palmer & Andersen 2008). Thus, the intriguing feature of the outward-facing membrane of frog skin, namely its large Na⁺ permeability and low K⁺ permeability (19), initiated biophysical studies that led to the discovery of the amiloride-blockable epithelial sodium ion channel (Lindemann & Van Driessche 1977), which turned out to constitute the entrance mechanism in several other types of NaCl-absorbing epithelia (Garty & Palmer 1997). This Na⁺ channel has been cloned and named ENaC (Canessa et al. 1994). The updated model with a paracellular shunt has been generalized to other transporting epithelia and has become the ‘definitive framework’ of epithelial organization (Reuss 2001), with the Na⁺/K⁺ pump being identical to the Na⁺,K⁺-ATPase, which was discovered by Jens Christian Skou (Skou 1957).

On the occasion of Hans Ussing’s receipt in 1999 of the A. N. Richards Award of the International Society of Nephrology, Gerhard Giebisch concluded his commentary with these words (Giebisch 1999):

At that critical time in the development of renal transport physiology, Hans and his associates provided us thus with a new conceptual basis for the study of tubule electrolytes and water transport. Hans’ novel concept of epithelial transport stimulated many of us to apply his ideas to renal physiology, leading to a true renaissance of transport phenomena at the single nephron level. There is hardly a single transport process in the kidney that has not been affected by the concepts that Hans and his associates have developed. A true renaissance in renal transport physiology was thus initiated by this novel way of defining transport phenomena. Hans’ work laid the basis of a major evolution in the understanding of membrane transport. As renal physiologists, we will always be deeply indebted to him.

Ussing received numerous academic honours, which are listed in his curriculum vitae below. The remarkable influence of his scientific work is illustrated by the invited papers of two dedicated issues of scientific journals: *Hans H. Ussing memorial issue: epithelial membrane transport* (*Journal of Membrane Biology*, vol. 184 part 3 (2001)) and *Epithelial ion transport—a tribute to Hans H. Ussing* (*Biochimica et Biophysica Acta*, vol. 1566, parts 1–2 (2002)).

**Curriculum Vitae**

1911 Born on 30 December in Soro, Denmark
1929 Graduated from high school in Sorø Academy with highest honours
1934 Cand. mag. in zoology with highest honours, University of Copenhagen
1934 Assistant Scientist, Zoophysiological Laboratory, University of Copenhagen
Hans Henriksen Ussing

1938 Dr phil., University of Copenhagen
1945 Amanuensis, Zoophysiological Laboratory, University of Copenhagen
1948 Rockefeller Fellow, the Donner Laboratory, University of California, Berkeley
1951 Extraordinary Professor in Zoophysiology, University of Copenhagen
1954 Guest Scientist, Institute de Biofisica, Rio de Janeiro
1955 Elected Member of Royal Danish Academy of Sciences and Letters
1957 Fogarty Scholar, National Institutes of Health, Bethesda, Maryland
1958 Walker-Ames Professor, University of Washington, Seattle
1958–81 Professor of Biochemistry, Head of Institute of Biological Chemistry, University of Copenhagen
1959 Honorary Member of American Physiological Society
1961 Member of Danish Academy of Technical Sciences
1964 Anders Jahre Medical Prize, University of Oslo
1964 Jens Rosenkjaer Prize, Copenhagen
1965 Dr med. honoris causa, University of Kiel
1965 Homer Smith Award, New York Heart Association
1966 Dr med. honoris causa, Louvain
1966 Honorary Member of Physiological Society, UK
1966–81 Chairman of the board of the Novo Foundation
1967 Honorary Member of the American Academy of Arts and Sciences
1969 Amory Prize, American Academy of Arts and Sciences
1969–75 Chairman of the Science Class, Royal Danish Academy of Sciences and Letters
1970 Dr scient. honoris causa, University of Cambridge
1971 Alfred Benzon Prize, Copenhagen
1973 Series of lectures, Centro de Investigacion y de Estrudio Avanzados del Instituto Politecnico Nacional, Mexico
1976 Series of lectures, Kiel University
1977 Dunham Lectures, Harvard University
1980 Ole Romer Medal, Royal Danish Academy of Sciences and Letters
1980 Palms of French Academy
1980 Fellow of National Academy of Sciences of United States
1981 Dr med. honoris causa, University of Copenhagen
1982 Novo Prize, Copenhagen
1984 Elected ForMemRS
1999 A. N. Richards Award
2000 Honorary Member of Royal Irish Academy
2000 Died on 22 December in the Herlev Hospital, Copenhagen

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Biographical Memoirs


