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Kirk L. Hamilton
Daniel C. Devor *Editors*

Basic Epithelial Ion Transport Principles and Function

Ion Channels and Transporters of Epithelia in Health and Disease - Vol. 1

Second Edition

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We dedicate this second edition to our families . . . Judy, Nathan, and Emma for KLH, and Cathy, Caitlin, Emily, and Daniel for DCD.

Preface to Second Edition—Volume 1

Our ultimate goal for the first edition of *Ion Channels and Transporters of Epithelia in Health and Disease*was to provide a comprehensive and authoritative volume that encapsulated the most recent research findings in basic molecular physiology of epithelial ion channels and transporters of molecular diseases from the laboratory bench top to the bedside. Additionally, we envisioned that the book would be very exciting and useful to a range of readers from undergraduate and postgraduate students, to postdoctoral fellows, to research and clinical scientists providing a wealth of up-to-date research information in the field of epithelial ion channels and transporters in health and disease. We firmly believe that the first edition fulfilled a niche that was crucially required. We have been informed that the first edition of the book has proven to be the best performing APS/Springer book based on downloaded chapters, to date. This is a direct testament to the world-class scientists and clinicians who contributed excellent chapters to that edition. Of course, there were many epithelial ion channels and transporters which were not included in the first edition, but certainly warranted inclusion.

With our second Edition, we have superseded our original expectations by increasing the number of chapters from 29 in the first edition to a 3-volume second edition including 54 chapters, resulting in 25 new chapters. All of the original chapters have been expanded. Again, we were very fortunate to recruit "key" outstanding scientists and clinicians who contributed excellent chapters, some of whom were unable to commit to the first edition. In the end, the second edition has a total of 128 authors from 13 countries across four continents and both hemispheres. We truly believe that this book series represents a worldwide collaboration of outstanding international scientists and clinicians.

Volume 1: Basic Epithelial Ion Transport Principles and Function

This is the first of three volumes highlighting the importance of epithelial ion channels and transporters in the basic physiology and pathophysiology of human diseases. This volume consists of 13 chapters (5 new chapters), including chapters focused on techniques used to study epithelial transport physiology, principles of epithelial transport function, the recent developments in the mathematical modeling of epithelia, the establishment and maintenance of epithelial polarity, protein sorting to specific membranes of epithelial cells, membrane protein folding, structure and endoplasmic reticulum-associated degradation, the fundamentals of transepithelial ion transport of chloride, sodium, and potassium, epithelial volume regulation, the fundamentals of bicarbonate secretion, and the role of non-coding RNA-dependent regulation of transport proteins. These chapters will set the "epithelial physiological" groundwork of the molecular participants, key concepts, and epithelial cell models that play critical roles in transepithelial ion transport function detailed throughout volumes 2 and 3 of this new edition.

It is our intent that the second edition continues to be the comprehensive and authoritative work that captures the recent research on the basic molecular physiology of epithelial ion channels and transporters of molecular diseases. We hope this new edition will be the "go-to" compendium that provides significant detailed research results about specific epithelial ion channels and transporters and how these proteins play roles in molecular disease in epithelial tissues.

As stated in the preface of the first edition, the massive undertaking of a book of this enormity would certainly be an "Everest" of work. We want to sincerely thank all of our authors, and their families, who have spared time from their very busy work and non-work schedules to provide exciting and dynamic chapters, which provide depth of knowledge, informative description, and coverage of the basic physiology and pathophysiology of the topic of their individual chapters.

We want to, again, thank Dr. Dee Silverthorn who planted the "initial seed" that developed into the first edition, which stemmed from a Featured Topic session entitled "Ion Channels in Health and Disease" held during the Experimental Biology meetings in Boston in April 2013 (chaired by KLH). Then, based on the performance of that edition, Dee "twisted" our arms, with love, to attempt a second edition in 2017. We, once again, want to extend our huge thanks, gratitude, and appreciation to the members of the American Physiology Society Book Committee for their continued faith in us to pursue such a monumental second edition.

As with the first edition, this 3-volume second edition would not have been possible without the excellent partnership between the American Physiological Society and Springer Nature and the publishing team in Heidelberg, Germany. Many thanks to Markus Spaeth, Associate Editor (Life Science and Books), and Dr. Andrea Schlitzberger, Project Coordinator (Book Production Germany and Asia), who guided us on our second book publication journey never dreaming that this edition would be a 3-volume book bonanza.

We extend special thanks to Anand Venkatachalam (Project Coordinator, Books, Chennai, India) at SPi Global who answered unending questions during the production process. We thank his production team who assisted us through the many stages of the publication of the second edition. We also thank Nancey Biswas (Project Management, SPi Content Solution, Puducherry, India), Nedounsejiane Narmadha (Production General, SPi Technologies, Puducherry, India), and Mahalakshmi Rajendran (Project Manager, SPi Technologies, Chennai, India) at Spi Global for their assistance for overseeing the production of the chapters during the final print and online file stages of the second edition.

We want to thank our mentors Douglas C. Eaton and the late Dale J. Benos for KLH; Michael E. Duffey and Raymond A. Frizzell for DCD; and our colleagues who guided us over the years to be able to undertake this book project.

Finally, and most importantly, we want to thank our families: Judy, Nathan, and Emma for KLH, and Cathy, Caitlin, Emily, and Daniel for DCD for all your love and support during this 8-year journey.

We dedicate this second edition to our families.

Dunedin, New Zealand — Kirk L. Hamilton
Pittsburgh, PA — Daniel C. Devor
July 2020

Preface

Ion channels and transporters play critical roles both in the homeostasis of normal function of the human body and during the disease process. Indeed, as of 2005, 16% of all Food and Drug Administration-approved drugs targeted ion channel and transporters, highlighting their importance in the disease process. Further, the Human Genome Project provided a wealth of genetic information that has since been utilized, and will again in the future, to describe the molecular pathophysiology of many human diseases. Over the recent years, our understanding of the pathophysiology of many diseases has been realized. The next great "step" is a combined scientific effort in basic, clinical, and pharmaceutical sciences to advance treatments of molecular diseases.

A number of unique ion channels and transporters are located within epithelial tissues of various organs including the kidney, intestine, pancreas, and respiratory tract, and all play crucial roles in various transport processes responsible for maintaining homeostasis. Ultimately, understanding the fundamentals of ion channels and transporters, in terms of function, modeling, regulation, molecular biology, trafficking, structure, and pharmacology, will shed light on the importance of ion channels and transporters in the basic physiology and pathophysiology of human diseases.

This book contains chapters written by notable world-leading scientists and clinicians in their respective research fields. The book consists of four sections. The first section of the book is entitled **Basic Epithelial Ion Transport Principles and Function** (Chapters 1–8) and spans the broad fundamentals of chloride, sodium, potassium, and bicarbonate transepithelial ion transport, the most recent developments in cell volume regulation, the mathematical modeling of these processes, the mechanisms by which these membrane proteins are correctly sorted to the apical and basolateral membranes, and protein folding of ion channels and transporters. The chapters in Section 1 provide the foundation of the molecular "participants" and epithelial cell models that play key roles in transepithelial ion transport function of epithelia detailed throughout the rest of this volume.

The second section is entitled **Epithelial Ion Channels and Transporters** and contains seventeen chapters (9–25) in which authors have concentrated their discussion on a particular ion channel or transporter ranging from chloride channels to the Na^+/K^+-ATPase, for example. Generally, the authors have initially provided a broad perspective of the physiology/biology of a particular ion channel or transporter in epithelial tissues, followed by a focused in-depth discussion of the latest physiology, cell biology, and molecular biology of the ion channel/transporter and then finish their discussion on aspects of pathophysiology and disease.

It will be appreciated following the discussion of the various ion channels and transporters that many of these transport proteins are potential pharmacological targets for possible treatment of disease. Therefore, the third section is entitled **Pharmacology of Potassium Channels** that consists of two chapters (26 and 27) that provide the latest developments on the pharmacology of calcium-activated potassium channels and small-molecule pharmacology of inward rectified potassium channels. It should be noted, however, that pharmacological information about various ion channels and transporters is also provided in some of the chapters found within Section 2 of this volume.

Finally, the last section in the book is entitled **Diseases in Epithelia** and consists of two chapters (28 and 29). These chapters are designed to bridge the basic cellular models and epithelial transport functions discussed throughout this volume with a compelling clinical perspective: from bench to bedside. In these chapters, Dr. Whitcomb discusses the role of ion channels and transporters in pancreatic disease, while Dr. Ameen and her colleagues similarly provide insights into the secretory diarrheas.

Our utmost goal, with this book, was to provide a comprehensive and authoritative volume that encapsulates the most recent research findings in the basic physiology of ion channels and transporters of molecular diseases from the laboratory bench top to the bedside. Additionally, we hope that the book will be very exciting and useful to a range of readers from students to research scientists providing a wealth of up-to-date research information in the field of epithelial ion channels and transporters in health and disease.

The undertaking of a book of this scale would always be a “mountain” of work. We want to give our heartfelt thanks to all of our authors who have taken time from their very busy work and non-work schedules to provide excellent chapters, which provided depth of knowledge, informative description, and coverage of the basic physiology and pathophysiology of the topic of their particular chapters.

We want to thank Dr. Dee Silverthorn who planted the “seed” that developed into this volume, which stemmed from a Featured Topic session entitled “Ion Channels in Health and Disease” held during the Experimental Biology meetings in Boston in April 2013 (chaired by KLH). We thank the members of the American Physiology Society (APS) Book Committee who had faith in us to pursue such an exciting book.

As with any book, this volume would not have been possible without the excellent partnership between the APS and Springer-Verlag and the publishing team at Heidelberg, Germany (Britta Mueller, Springer Editor, and Jutta Lindenborn, Project Coordinator). We wish to thank Portia Wong, our Developmental Editor at

Springer+Business Media (San Mateo, CA), and her team who assisted with the early stages of the publishing process that greatly added to this contribution. Finally, special thanks to Shanthi Ramamoorthy (Production Editor, Books) and Ramya Prakash (Project Manager) of Publishing—Springer, SPi Content Solutions—SPi Global and their production team who assisted us through the final stages of the publication of our book.

Finally, we want to thank our mentors Douglas C. Eaton and the late Dale J. Benos for KLH; Michael E. Duffey and Raymond A. Frizzell for DCD; and our colleagues who guided us over the years to be able to undertake this volume.

Dunedin, New Zealand — Kirk L. Hamilton
Pittsburgh, PA — Daniel C. Devor
June 2015

Contents

About the Editors

Kirk L. Hamilton was born in Baltimore, Maryland, in 1953. He gained his undergraduate (biology/chemistry) and M.Sc. (ecology) degrees from the University of Texas at Arlington. He obtained his Ph.D. at Utah State University under the tutelage of Dr. James A. Gessaman, where he studied incubation physiology of Barn owls. His first postdoctoral position was at the University of Texas Medical Branch in Galveston, Texas, under the mentorship of Dr. Douglas C. Eaton where he studied epithelial ion transport, specifically the epithelial sodium channel (ENaC). He then moved to the Department of Physiology at the University of Alabama, Birmingham, for additional postdoctoral training under the supervision of the late Dr. Dale J. Benos where he further studied ENaC, and non-specific cation channels. He took his first academic post

in the Department of Biology at Xavier University of Louisiana in New Orleans (1990–1994). He then joined the Department of Physiology at the University of Otago in 1994, and he is currently an Associate Professor. He has focused his research on the molecular physiology and trafficking of potassium channels (specifically KCa3.1). He has published more than 60 papers and book chapters. His research work has been funded by the NIH, American Heart Association, Cystic Fibrosis Foundation, and Lottery Health Board New Zealand. Dr. Devor and he have been collaborators since 1999. When he not working, he enjoys playing guitar (blues and jazz) and volleyball. Kirk is married to Judith Rodda, a recent Ph.D. graduate in spatial ecology. They have two children, Nathan (b. 1995) and Emma (b. 1998).

Daniel C. Devor was born in Vandercook Lake, Michigan, in 1961. His education took him through the Southampton College of Long Island University, where he studied marine biology, before entering SUNY Buffalo for his Ph.D., under the guidance of Dr. Michael E. Duffey. During this time, he studied the role of basolateral potassium channels in regulating transepithelial ion transport. He subsequently did his postdoctoral work at the University of Alabama, Birmingham, under the mentorship of Dr. Raymond A. Frizzell, where he studied both apical CFTR and basolateral KCa3.1

in intestinal and airway epithelia. He joined the University of Pittsburgh faculty in 1995 where he is currently a Professor of Cell Biology. During this time, he has continued to study the regulation, gating, and trafficking of KCa3.1 as well as the related family member, KCa2.3, publishing more than 50 papers on these topics. These studies have been funded by the NIH, Cystic Fibrosis Foundation, American Heart Association, and pharmaceutical industry. When not in the lab, he enjoys photography and growing exotic plants. Dan is married to Catherine Seluga, an elementary school teacher. They have three children, Caitlin (b. 1990), Emily (b. 1993), and Daniel (b. 1997).

Chapter 1
Techniques of Epithelial Transport Physiology

Kirk L. Hamilton

Abstract Epithelial tissues play many roles in maintaining homeostasis of the human body. These tissues separate the body from the external environment (e.g., skin which protects the body), and of course, epithelial tissues separate body compartments, line the surfaces of organs, and line the inner surfaces of many hollow organs. Epithelial cells are polarized as there are specific transport proteins (ion channels and ion transporters) residing in the apical and basolateral membranes of the epithelial cells. Different epithelial cells perform specific functions in the regulation of absorption and secretion of ions, solutes, nutrients, and water. Understanding how these tissues (cells) function has been challenging and a number of techniques have been developed and/or adapted to study the functions of epithelial tissues and cells. Our ability to understand the physiology and the disease pathophysiology of epithelial tissues and cells is really reduced down to determining the fundamental characteristics and basic biology/physiology of the specific ion channels and ion transporters participating in overall epithelial transport physiology. This chapter provides a historical overview of various experimental techniques which have been instrumental and are still employed to discover intriguing aspects of epithelial ion transport physiology.

Keywords Epithelial transport · Ussing chamber · Radioisotopic studies · Short-circuit current · Micropuncture · Isolated perfused-tubule · Evert-sac preparation · Brush border vesicles · Site-directed metagenesis · PCR · Fluctuation analyses

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1.1 Introduction

Epithelial tissues are vitally important for proper homeostasis of the body. Our understanding of epithelial tissues can be pared down to the proper function of individual epithelial cells. These cells are polarized and maintain specific ion channels and transporters on the apical and basolateral membranes of the cell which are required for transepithelial ion, solutes, nutrients, and water transport (see Chaps. 2 and 3 of this volume for discussions about epithelial cell structure, functions, and the establishment of epithelial polarity). Currently, over 70% of the Federal Drug Administration-approved drugs are targeted to membrane proteins including ion channels, ion transporters, and receptors (Mushegian 2018). Therefore, determining the basic biology/physiology of epithelial ion channels and transporters is crucial in making the leap from basic epithelial function to understanding the pathophysiology of diseases of epithelial tissues.

As science progresses, scientists' ingenuity results in the development of experimental methods, techniques, and equipment that furthers scientific creativity. The great Danish physiologist, August Krogh, who received the Nobel Prize in Physiology or Medicine in 1920 for 'his discovery of the capillary motor regulating mechanism', stated that 'While it is undoubtedly true that the chief tool and weapon in research is thought and ideas and that a large amount of experimental work in biology is more or less wasted for lack of thought, it is less true that progress depends to a very large extent upon methods and that new methods may open up new and fruitful fields' (Krogh 1937a). He wrote those words in the context of the use of isotopes as indicators in biological research. Still, those words are appropriate even now when discussing the various experimental techniques that many scientists use today in their research. Of course, there are many techniques that are the backbone of molecular benchtop science. However, when envisioning studying biology/physiology at the animal, organ, tissue or cell level, additional technical approaches are required. With respect to epithelial tissues, one conducts experiments at the tissue and/or cell level to describe and confirm physiological phenomena. This chapter describes the historical concepts and technical aspects of commonly used methods for studying transport physiology of epithelial tissues and cells.

1.2 The Road to Epithelial Transport: How Did Epithelial Ion Transport Begin?

1.2.1 Early Research in Epithelial Transport

The age of epithelial transport physiology has had a long journey that began in the early nineteenth century. As early as 1826, Dutrochet (as cited in Reid 1890) reported that the cecum (containing milk) of the fowl, when placed in water, gained weight over a 36-h period suggesting the movement of water by osmosis.

Subsequently, Matteucci and Lima (1845), using frog skin, determined that the speed of 'osmotic transference of fluid' varied depending upon the orientation of the skin with respect to the bathing solutions, and that this difference was observed while the skin was freshly removed from the animal (Reid 1890). In their paper, Matteucci and Lima confirmed those same findings in the gastric mucous membrane of the lamb, cat, and dog. Therefore, even in the first quarter of the nineteenth century, scientists were intrigued with understanding the transport of water across epithelial tissues.

Stanley Schultz (1989) in his poignant review reminded the epithelial transport community of the work by E. Waymouth Reid. Schultz stressed that the contributions of Reid had not been fully appreciated during most of the twentieth century, although Reid actively investigated the field of epithelial transport physiology in the 1890s and early 1900s. His studies of frog skin osmosis (1890) and absorption without osmosis in the rabbit intestine (1892b) and the frog skin (1892a) confirmed the results of Matteucci and Lima (1845). From his own experimental results, Reid described four notable observations that have withstood the test of time for nearly 130 years. These observations are:

1. '. . .The normal direction of easier osmotic transference of fluid through the living skin of the frog is in the direction of outer to inner.'
2. '. . .The transference of fluid through the skin in the above direction is intimately associated with the physiological conditions of its tissues; conditions or agents tending to depress vitality diminish the transfer in the normal direction, while stimulants give rise to augmentation.'
3. '. . .The cause of the easier transference of fluid from the outer towards the inner surface, is probably to be found in the existence of an absorptive force dependent on protoplasmic activity, and comparable to the secretive force of the gland cell.'
4. Finally, '. . .In consequences of the absorptive force acting from without inwards, an alteration of the relations of the surfaces of the skin to the two fluids used in an osmosis experiments modifies the rapidity of the transfer of fluid from one to the other side of the membrane, according as the force exerted by the living tissues is with or against the osmotic stream.' (Quoted from Reid 1890.)

Additionally, Reid followed his earlier work with studies of fluid transport in various epithelia including the cat and rabbit ileum (inverted gut prep), gastric mucosa of the toad, and frog skin (Reid 1901). Indeed, Schultz (1989) revered the work of Reid by stating that '. . .Reid had. . .for the first time unambiguously demonstrated and recognized "active transport"'. As with the famous quote by Sir Isaac Newton '. . .If I had seen further than others, it is by standing on the shoulders of giants'; this is true for scientists today whom have learned and built upon the tremendous work of Dutrochet, Matteucci, Lima, and Reid.

It was, however, Krogh and de Veresy who were early innovators in the era of membrane physiology because of their radioactive isotopic studies; followed shortly by Hans Ussing and his famous 'chamber' and the short-circuit current technique (Schultz 1989).

1.3 Radioisotopes and Radioisotopic Tracers Studies

1.3.1 Early Pioneer Researchers in Radioactivity

When one ponders about radiation or radioactivity several names quickly spring to mind including Wilhem Röntgen, Henri Becquerel, and Marie and Pierre Curie. To begin a discussion of radioisotopes, we must go to the beginnings of the work by Röntgen who is credited with the discovery of 'X-rays' in 1895 (Röntgen 1895; Posner 1970). Röntgen was experimenting with different types of vacuum tubes including cathode-ray tubes when he discovered a glow on a black cardboard screen coated with fluorescent material in the distance away from the cathode ray (Posner 1970). Röntgen surmised that the resulting fluorescence could only have originated from one end of the cathode ray tube (Lentle and Aldrich 1997). The contribution of Röntgen's 'invisible ray' to the medical field has been incredible. Röntgen was awarded the 1st Nobel Prize in Physics in 1901 'in recognition of the extraordinary services he has rendered by the discovery of the remarkable rays subsequently named after him'.

In 1896, Becquerel had been wondering if phosphorescent material could emit similar 'invisible rays' (Becquerel 1903). He believed that uranium salts might be the suitable 'matter' for his investigations. Within a short period, after some key experiments, Becquerel soon realized that uranium salts were 'the source' of the emitted rays causing this phenomenon; essentially identifying 'radioactivity'. Becquerel published six scientific papers about his research on radioactivity in 1896 which are described by Myers (1976).

Marie Curie was intrigued with Becquerel's work on uranium and believed that other elements must emit these 'new Becquerel rays' (Curie 1905; Hevesy 1961). She began her quest for other elements exhibiting 'rays', and in 1898, she identified that thorium emitted rays. Then, she focused on minerals containing uranium including pitchblende (now known as uraninite) and torbernite (also known as chalcolite), and discovered that the intensity of radiation emitted by these minerals was much greater than uranium (Hevesy 1961). Also in 1898, Marie and Pierre Curie reported the discovery of the radioactive element polonium. Later that same year, the Curies reported the discovery of radium (Curie 1905; Hevesy 1961). Marie Curie continued her work on polonium and radium and was determined to isolate these elements. By 1910, she successfully isolated radium metal from pitchblende, but was never able to isolate polonium.

For their collective work on radioactivity, Becquerel shared the Nobel Prize in Physics in 1903 with Marie and Pierre Curie. Becquerel was awarded the Prize 'in recognition of the extraordinary services he has rendered by his discovery of spontaneous radioactivity'; while the Curies' received the Prize 'in recognition of the extraordinary services they have rendered by their joint researches on the radiation phenomena discovered by Professor Henri Becquerel'. Based on her work and the isolation of radium and attempts to isolate polonium, Marie was awarded the Nobel Prize in Chemistry in 1911 'in recognition of her services to

the advancement of chemistry by the discovery of the elements radium and polonium, by the isolation of radium and the study of the nature and compounds of this remarkable element'. Interested readers are referred to Curie (1911) for her Nobel lecture.

How did radioactive elements aid our understanding of epithelial ion transport function?

1.3.2 Taking Radioisotopes Tracers into Chemistry and Biology: George de Hevesy

The application of radiotracers to physical chemistry and biology started shortly after the early pioneering work of the scientists mentioned in the previous section. George de Hevesy (referenced Hevesy in scientific publications) is credited as the first scientist to use isotopes as a tracer in biological studies in 1923 (Hevesy 1923; Levi 1985). However, earlier work commenced when Hevesy visited with Ernest Rutherford (Nobel Prize in Chemistry in 1908 'for his investigation into the disintegration of the elements, and the chemistry of radioactive substances') in 1911.

During 1912–1915, Hevesy began experimenting with mixtures of lead in the form of nitrate in water and adding a negligible amount of radium D and determined the radioactivity with an electroscope. An electroscope is an instrument which detects electrical charges (electrical potential) of an object, especially as an indication of the ionization of air by radioactivity. Hevesy noted that radioactivity was detected in the origin fraction of the lead nitrate. Later, Hevesy stated that that initial finding was the first hint of the potential power of what would become radio-labelled tracer studies (de Hevesy 1944). The next step was to explore this methodology in a biological application context.

Hevesy did not settle for working only in physical chemistry and, therefore, branched out into biological systems. In 1923, Hevesy published the first paper using a radioactive indicator in biological research (Hevesy 1923). In that paper, he reported the absorption and translocation of lead in horse-bean (*Vicia fiba*) plants. He used the element thorium B as the radioactive indicator of lead, and clearly demonstrated that lead was taken up by the roots and distributed throughout the plant. Hevesy also determined that the ordinary lead could displace the thorium B suggesting that the 'radioactive lead' was not incorporated within the bean plant. Krogh's (1937a) thoughts about results of de Hevesy's were that '. . .lead atoms are in reality never fixed anywhere, but are always on the move up and down the plant to and from the single cells, to and from the organic lead compounds which are continually formed and reformed.'

Hevesy continued his research on isotope tracer studies with deuterium in humans (Hevesy and Hofer 1934) and in frogs (Hevesy et al. 1935), and with phosphorus in rabbits (Hahn et al. 1937). Indeed, Krogh (1937a) applauded Hevesy and wrote that Hevesy '. . .was the first to see the possibilities offered in biology by recognizable

isotopes and made the classical and fundamental experiments with radioactive lead'. Additional information and the use of radioactive tracers in experimental studies investigating ion transport in epithelia is found in Sect. 1.4.1.

Hevesy was the recipient the 1943 Nobel Prize in Chemistry 'for his work on the use of isotopes in the study of chemical processes'. For further information about Hevesy's scientific contributions, readers are directed to de Hevesy (1944), Cockcroft (1967), Levi (1985), and Niese (2006).

For those interested in learning more about early days of radioisotopes and radiotracers, and the use of these molecules in application to biological studies, readers are directed to the following excellent reviews by Krogh (1937a, b), Hevesy (1940, 1962), de Hevesy (1944), Popják (1948), Lyon (1949), Burris (1950), Cockcroft (1967), Posner (1970), Nitske (1971), Ussing (1980), Lentle and Aldrich (1997), Niese (2006), and Creager (2013).

1.4 The Epithelial Cell Begins to Open Up: Hans H. Ussing, the 'Black Box', the Ussing Chamber, and I_{sc}

It is interesting to observe how the scientific endeavors of one individual can affect/change the course of an entire research field. Once such person was Hans Henriksen Ussing (1911–2000) whose pioneering work in ion transport physiology, and his development of innovative research tools and methods paved the way for epithelial transport physiologists/scientists to enter the physiological 'Black Box' of the epithelial cell (Larsen 2002, 2009). The monumental work of Ussing, beginning in the mid-1930s, which includes the Ussing chamber and the short-circuit current (I_{sc}) technique (discussed below), ion flux experiments, and ion exchange experiments, that undoubtedly, ushered in the 'age of epithelial ion transport physiology' as we know it (Ussing 1949a; Ussing and Zerahn 1951). Schultz (1989) claimed that it was Ussing, his work, and his conceptual cell model had set '. . .the birth of the modern era of epithelial transport physiology with the introduction of the Koefoed-Johnsen-Ussing (KJU) double membrane model' (Koefoed-Johnsen and Ussing 1958). The use of the Ussing chamber technique is still thriving today in countless numbers of epithelial physiology laboratories in both the academic and pharmaceutical environments throughout the world (Lenneräs 2007).

Others have stated the 'impact' of Ussing's work on science, and, in particular, epithelial ion transport physiology. Schultz (1998) wrote that Ussing's work of 1951 was '. . .his crowning triumph with Zerahn. . .[Ussing and Zerahn 1951]. . .the demonstration of the equivalence between the short-circuit current and active Na^+ transport across isolated frog skin. Na^+ could be transported across viable frog skin from the outer bathing solution to an identical inner bathing solution in the absence of external driving forces.' Schultz (1989) further declared that 'Ussing's demonstration of active transport was immediately embraced, and a new paradigm was born.' Lindemann (2001) exemplified Ussing's contribution to science simply

with four words '...founder of epithelial transport...' Similarly, Palmer and Andersen (2008) paid homage to Ussing by writing 'In a sense the field of epithelial polarity began in 1958 with the Koefoed-Johnsen and Ussing paper.' (Koefoed-Johnsen and Ussing 1958). Clarke (2009), while describing the application of the Ussing chamber technique in intestinal tissues, acknowledged that 'As our understanding of the molecular interactions of transporters is refined, the methodology of the Ussing chamber will continue to provide a "gold standard" in the application of this knowledge to the physiological complexities of healthy and diseased intestinal mucosa'.

Finally, Jerrold Turner, who was the recipient of the 2015 'Hans H. Ussing Distinguished Lectureship' of the Epithelial Transport Group of the American Physiological Society, summed up his thoughts about Ussing's contribution to the field of epithelial transport physiology as follows, 'The great strides forward in our understanding of epithelial transport in the relatively short time since Ussing created his chamber are, nevertheless, remarkable. And, as is often the case, this knowledge has led to even more exciting frontiers for exploration.' (Hermann and Turner 2016). One could list more quotes from other eminent scientists who have highlighted Ussing's life and work with equally superlative remarks. How did Ussing change the epithelial transport world?

1.4.1 Ussing's Early Years in Preparation for His Chamber

Ussing's journey to epithelial transport physiology and the Ussing chamber was not a straight-forward pathway. He studied biology and geography at the University of Copenhagen (UCph) and graduated with a Master's in 1934. He also studied zoology, biochemistry, physiology, and physical chemistry (Larsen 2009). All these subjects would serve Ussing well over his 65+ year research career. During the summer of 1933, Ussing worked as a marine biologist and a hydrographer with the *Lauge Koch's* 3-year expedition to East Greenland, and he collected and analyzed zooplankton samples (Ussing 1980). He received additional samples for the remainder of the expedition which provided him with his research material culminating with his D. Phil. in 1938 from the UCph (Ussing 1980; Larsen 2002, 2009). One of the examiners of his doctorate thesis was August Krogh who thought very highly of Ussing (Larsen 2002). Krogh would play a major role and influence in Ussing's research future.

In 1935, Ussing joined Krogh at the Zoophysiological Laboratory at UCph at an auspicious time when Krogh had begun working with Niels Bohr (awarded the Nobel Prize in Physics in 1922 'for his services in the investigation of the structure of atoms and of the radiation emanating from them'), and Hevesy discussing radioisotopic studies. Earlier, Krogh had visited Harold C. Urey who had recently discovered 'heavy hydrogen' and followed that with heavy water, deuterium, D_2O (Urey 1925, 1933; Washburn and Urey 1932; Larsen 2009). Urey received the Nobel Prize for Chemistry in 1934 'for the discovery of heavy hydrogen' (Urey 1935). Krogh had

asked Urey for some heavy water for proposed experiments to determine the permeability of living membranes using D_2O as a tracer for normal water (Ussing 1980; Larsen 2002, 2009). It was Hevesy who suggested the idea of using radioisotopes as tracers to examine biological processes (Hevesy 1923; Ussing 1980; Larsen 2002). The team was ready! Krogh, along with Hevesy and Hofer used D_2O to examine the water permeability of the frog skin and demonstrated that water movement was passive, although there was an inconsistency that normal water had a higher permeability than D_2O (Hevesy et al. 1935; Ussing 1980; Larsen 2002). For further details regarding the early historical aspects of radio-isotopic studies by the Copenhagen group, the reader is directed to Larsen (2002) and (2009).

During that period, Ussing noted that D_2O disappeared from the water and was incorporated into tissues, possibly into proteins (Ussing 1980). At that time, Ussing was a young member of the laboratory and was quite interested in biochemistry, therefore, he decided to pursue the use of D_2O in examining incorporation into amino acids and proteins (Ussing 1980). He quickly succeeded in developing new methods and protocols for measuring the incorporation of D_2O into amino acids and reported that D_2O-labelled amino acids were introduced into certain body proteins very quickly, both in mice and rats (Ussing 1938a; Larsen 2009). Larsen (2002) claimed that Ussing had '. . .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.' Further, Larsen continued by stating that Ussing '. . .demonstrated how tracer technology provides fundamentally new opportunities for exploring the dynamic state of living cells.' Ussing continued his pursuit of protein biochemistry as evidenced by his series of seminal publications during the late 1930s and the 1940s (Ussing 1938a, b, 1941, 1943a, 1945a, b, 1946).

In the mid-1940s, Krogh, as a prominent Danish citizen, was advised to flee to Sweden due to the German occupation of Denmark, so he asked Ussing to oversee the radiotracer program, which Ussing accepted, although reluctantly (Larsen 2009; Larsen, Pers. Comm.). Krogh suggested that Ussing should focus on the transport of K^+ in the frog muscle. However, Ussing chose to examine the Na^+ transport instead (Ussing 1980; Larsen 2002). Ussing quickly published studies on the exchange of radioisotopic tracers in frog muscle tissue (Ussing 1947; Levi and Ussing 1948).

However, what about Ussing and epithelial transport? Throughout Ussing's time with Krogh, there had been work performed with frogs by the group (Hevesy et al. 1935; Krogh 1937b), and even Ussing had conducted experiments with amphibians (axolotl, *Ambystoma mexicanum*; Barker Jørgensen et al. 1946) and red blood cells (Ussing 1943b). Ussing realized that with the success of axolotls in which he demonstrated uptake of radioactive Na^+ (that is, $^{24}Na^+$) through the skin of animals in salt balance (Barker Jørgensen et al. 1946; Larsen 2002), the skin of amphibians would be the preparation to continue his studies. Ussing (1980) stated that 'I decided to begin with the isolated frog skin, mostly because I found it easier to skin a frog than an axolotl but also because Krogh had demonstrated that frogs take up both sodium and chloride from very dilute solution.' The epithelial transport world changed, forever, and Larsen (2009) reminded us that the '. . .frog skin became the

preparation of choice of Ussing [and many others], which continued to challenge him for more than 50 years.' Ussing's nearly 20 years of experience with radioisotope tracer, ion exchange, and ion flux experiments, undoubtedly, gave him an excellent background in transport physiology before focusing his efforts on epithelial transport physiology.

1.4.2 The Ussing Chamber and the Short-Circuit Technique

In 1948, Ussing received a Rockefeller Scholarship and journeyed to the Donner Laboratory at the University of California at Berkeley. Shortly after arriving in Berkeley, he was introduced to Lund and Stapp's (1947) book entitled *Bioelectric Fields and Growth*. Ussing read the book and commented that someone '. . .had attempted to draw the electric current from frog skins via reversible lead-lead chloride electrodes.' (Ussing 1980). Ussing further stated 'When I recalculated the currents drawn from frog skins in terms of sodium fluxes they turned out to be roughly the same order of magnitude as isotope fluxes I had measured. . . . a plan took shape: If one could "short-circuit" the skin via suitable electrodes so that the potential drop across was reduced to zero and if the bathing solutions were identical then only actively transported ions could contribute to the current passing the skin; the flux ratio for passive ions would become one.' So, the idea of the I_{sc} technique was already germinating. This was the basic electrical 'workings' of the short-circuit current in which Ussing would use an external current to drive the transepithelial current generated by the frog skin back to zero, thus, determining the current of the actively transported ions.

Once deciding upon the frog skin, Ussing began applying the radiotracer technique to the frog skin and examined the ion transport across the flux of $^{24}Na^+$ and $^{38}Cl^-$ across the skin (Ussing 1949a). For that project, he developed an apparatus (Fig. 1.1) which was comprised of separate compartments in which the skin was isolated between two bathing solutions on either side of the frog skin. From that study, he demonstrated that the net Na^+ flux was from outside to inside and that was higher than the outflow of Na^+; he reported the pH dependence for Na^+ flux, and that the Cl^- influx was lower than Na^+ at times, but there was a large parallelism between the potential difference across the skin and the influx of Na^+ and Cl^- (Ussing 1949a). With this chamber, Ussing quickly reported the resting potential and ion movements of the frog skin (Levi and Ussing 1949), and using I^{131}, they demonstrated that inward movement of I was less than the outward movement and that this transport was not active (Ussing 1949b). However, after those studies, Ussing was eager to return to his earlier ideas of examining the 'electrical properties' of the frog skin. Although the chamber and technique used in those recent experiments were just a foreshadow of what was in store for the epithelial transport world through the rest of the twentieth century and still a prominent technique in the twenty-first century. . . . . here comes the famous paper of 1951!

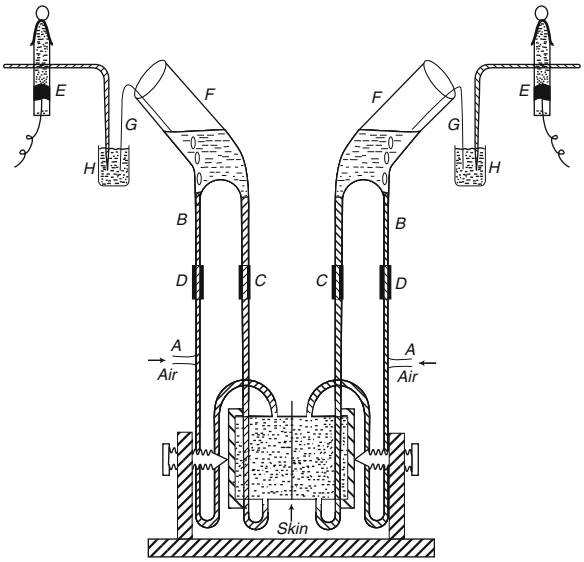

Fig. 1.1 This is a diagram of the experimental apparatus developed by Ussing. The solutions in the two chambers thus formed on either side of the skin are circulated by blowing air or any gas mixture wanted through the side tube. A. The solutions will then ascend to the funnels F through the tubes B and return to the chambers through the tubes C. From the funnels F samples can be drawn. If the total contents of for instance the inside circulation system is to be removed, an arterial clamp is placed on the rubber tube D. Then the pressure air cannot escape that way and will force the solution in the chamber up into the funnel. From here the solution is removed with a pipette or with the suction pump. The funnels are further used for making contacts between the solutions and two calomel electrodes E. The contacts are made through the capillary tubes G. One end dips in the funnel and the other end in a small tube H filled with saturated KCl solution. Before a measurement of the potential difference is made, H is lowered and the solution is allowed for a moment to flow through G before H is elevated again just so much that a little KC1 solution penetrates into the end of the capillary [from Ussing (1949a), with permission from John Wiley & Sons, Inc., UK]

Prior to Ussing's work on the frog skin, others had laid down a foundation of information about the frog skin. Francis and Pumphrey (1933) wrote that the concept of a skin potential had been reported in the 1850s. Larsen communicated to this author that Dubois Raymond (1848) suggested the phenomena of a skin potential (Larsen, Pers. Comm.). Lund (1926) had demonstrated a cyanide concentration-dependent reversible decrease of the electrical polarity ('difference in electrical potential' as stated by Lund) of the isolated frog skin. Additionally, Francis (1933) and Francis and Pumphrey (1933), using calomel electrodes, examined the electrical properties and the resting skin potential of the isolated frog skin. Francis (1933) determined that the potential increases as the temperature was raised to 20°C and fell above that temperature. Furthermore, they demonstrated that oxygen deprivation rapidly reduced the potential and that removing Ca^{2+} and K^{+} from the solutions resulted in a similar effect (Francis and Pumphrey 1933). Others have used frog skin

to conduct experiments examining the resting potential and currents of the skin and the effects of changing ion concentrations across the skin (Stapp 1941; Meyer and Bernfeld 1946). Ussing was aware of these studies. However, and more importantly, Ussing made the 'major link' between electrical current and the active movement of specific ions across the frog skin. These ideas culminated in the famous research papers of 1951 by Ussing and Zerahn, and in 1958 by Koefoed-Johnsen and Ussing.

Before the 1951 paper, Ussing had reported that the influx of Na^+ transport across the frog skin was higher than the outflux of Na^+ using two symmetrical skin preparations for radioactive influx and efflux studies, respectively, and both monitored by the $^{24}Na^+$ isotope (Levi and Ussing, 1949). With the introduction of his modified chamber and rig from that used in 1949, Ussing and Zerahn (1951) were now able to implement the short-circuit current technique in parallel with previous used radiotracer methods (Fig. 1.2). This chamber provided the ideal apparatus for the cells of an epithelial tissue to be studied in a precisely defined way. Again, the isolated frog skin epithelium was mounted between two fluid-filled chambers with essentially the same design as still used today. Hence, the introduction of the Ussing chamber opened a vast new opportunity to study ion transport of epithelial tissues. Ussing enlisted the help of Karl Zerahn to aid in the wiring of the circuit to be able to short-circuit the tissue and Ussing made the glass chambers (Ussing 1980) (Fig. 1.2). They conducted the first investigation of ion transport physiology (Na^+, in this case) of an epithelium in which radioisotopes (Na^{24}) could be used while simultaneously monitoring the 'active Na^+ current' generated by the frog skin.

As one reads the 1951 paper, one realizes there are five central advances made by Ussing and Zerahn. (1) They applied the short-circuiting (I_{sc}) technique to an epithelial tissue preparation; (2) They described an apparatus (the Ussing chamber with an associated DC-current generator, i.e., a battery) that permitted them to measure, simultaneously, the electrical current and the net Na^+ flux through the frog skin; (3) They bathed the frog skin with identical solutions (NaCl Ringer's solution) on either sides of the epithelium, thus preventing transepithelial passive net-ion flows. Thus, under those experimental conditions, ions that move by active transport would continue, and the generated short-circuit current would result from a net transport of those ions; (4) They stated that one could calculate the electromotive force for Na^+ and also the resistance to the Na^+ current from the efflux of Na^+ and the I_{sc} current. Lastly, Ussing and Zerahn reported that the net active transport of Na^+ resulted in the I_{sc} current generated by the frog skin, and this was based on the ^{24}Na experiments compared with the I_{sc} current measurements. Therefore, Na^+ influx across the entire frog skin epithelium (from pond water to the blood) dominated the Na^+ efflux with little Na^+ being transported from the blood to the pond water. The cellular model of Na^+ absorption is reviewed in other chapters of this volume and Volume 3 (Chaps. 8 and 9 of this volume and Chap. 18 of Volume 3) of this series, thus, the 1958 paper will not be discussed here. Interested readers are directed to Palmer and Andersen (2008) who celebrated and highlighted the 50th anniversary of the Koefoed-Johnsen and Ussing 1958 paper.

Today, the Ussing chamber method has been applied to virtually every epithelium in the animal body, including the reproductive tract, exocrine/endocrine ducts,

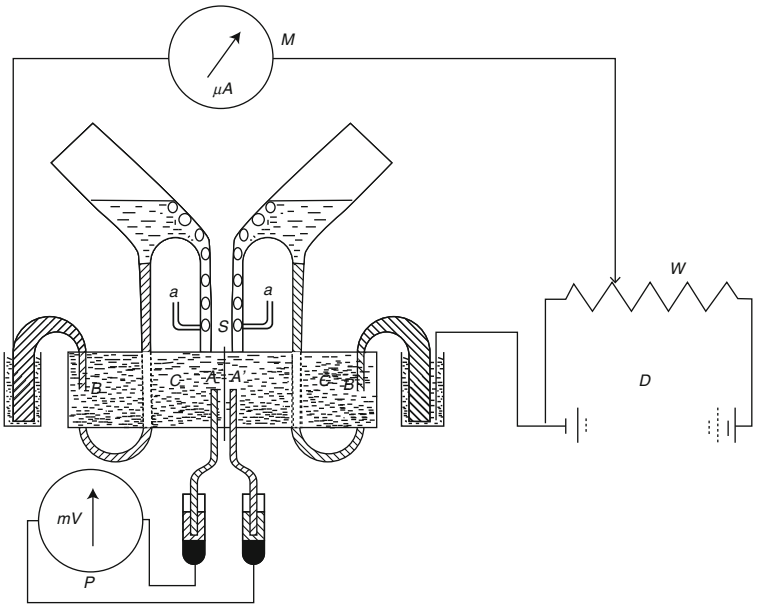

Fig. 1.2 Diagram of the chamber apparatus used by Ussing and Zerahn (1951) which was modified from chamber in Ussing's (1949a) paper. The apparatus used for determining Na flux and short-circuit current is shown diagrammatically in Fig. 1.2. The skin S, is placed as a membrane separating the Ringer solutions in the celluloid tubes C. Two narrow agar-Ringer bridges A and A4′ open on either side in the immediate vicinity of the skin. The outer ends of A and-A′ make contact with saturated KC1-calomel electrodes. The potential difference between the latter is read on the tube Potentiometer, P. Another pair of agar-Ringer bridges, B and B′ open at either end of the celluloid tubes, as far as possible from the skin. The outer ends of these bridges dip into beakers with saturated KC1 saturated with AgC1. Spirals of stout silver wire, immersed in these beakers, are used as electrodes through which an outer E. M. F. can be applied. The voltage is supplied from the dry battery D, and by aid of the potential divider, W, the voltage is adjusted so that the potential difference across the skin, as read on the potentiometer P, is maintained equal to zero. It is obvious that this is equal to a total short-circuiting of the skin potential. The current passing the skin at zero potential difference or any other potential difference desired is read on the microamperemeter M. Usually readings every five minutes suffice for a sufficiently accurate graphical integration, over the period between two Na^{24} samples, of the total amount of electricity crossing the skin. The potential was, however, under continuous observation during the experiments, so that also unexpected changes in current strength could be recorded, should they occur [from Ussing and Zerahn (1951), with permission from John Wiley & Sons, Inc., UK]

intestine, respiratory airway, eye, and choroid plexus. Furthermore, the method has been extensively used for studies of cultured epithelial cells (primary cells and stably transfected cell lines) where tight junction integrity maintains apical and basolateral membrane polarity.

As noted above, and will be presented further in this chapter, the discoveries of x-rays, the elements radium and polonium, heavy water, the development of research tools (site-directed mutagenesis and PCR) and techniques (e.g., patch-clamp technique, Sakmann and Neher, Nobel Prize for Physiology or Medicine, 1991 for their

discoveries concerning the function of single ion channels in cells) which have ushered in new 'eras' of research have garnered Nobel Prizes. Considering the major impact of Ussing's work in the field of epithelial transport physiology, was he ever nominated for a Nobel Prize? Indeed, Ussing was nominated at least once. Erik Larsen informed this author that a group from Copenhagen led by Ulrik Lassen and Ove Sten-Knudsen with supporting letters from the USA, UK, and Germany was submitted to Stockholm in the 1970s for consideration for a Nobel Prize. Unfortunately, even with an extraordinarily strong case for Ussing, the nomination was unsuccessful (Larsen, Pers. Comm.).

For more information regarding the discoveries of Hans Ussing, see the Hans Ussing Memorial issue of the *Journal of Membrane Biology*, Issue 3 Vol. 184, 2001 (https://link.springer.com/journal/232/184/3/page/1). The following are reviews that may be of use to interested readers; Ussing et al. (1974), Ussing (1980), Lindeman (2001), Larsen (2002), Palmer and Andersen (2008), Clarke (2009), Larsen (2009), Hamilton (2011), Hermann and Turner (2016), and Zajac and Dolowy (2017).

1.5 The Micropuncture Technique

Prior to the Ussing's work, the quest to acquire scientific information about epithelia transport function was certainly flourishing as noted in the earlier sections of this chapter. When trying to understand the function of a complex organ such as the kidney, one must step back and think about the basic structure of the organ. As we know, each kidney is composed of ~1×10^6 nephrons, which are the function units of the kidney. In the first quarter of the twentieth century, Wearn and Richards revolutionized the study of the kidney by introducing the micropuncture technique in 1924. This technique truly opened the kidney for scientific exploration.

1.5.1 Historical Aspects of the Micropuncture Technique

Starting to unravel the function of an organ (e.g., the kidney) or even a small structure (e.g., the nephron) within an organ presented a major technical challenge for scientists. Hence, advancement of knowledge is driven by the development of new technology. A case in point is our early understanding of the function of the single nephron of the kidney. In their 1924 seminal paper, Wearn and Richards established the renal micropuncture technique that allowed them to puncture surface nephrons of the frog kidney with pipettes to determine the composition of the ultrafiltrate. Some 80 years later, Sands (2004) stated that 'The development of the micropuncture by Wearn and Richards in 1924 ranks as one of the greatest advances in renal physiology during the 20th century.' Lorenz (2012) and others (Sands 2004; Vallon 2009) remarked that the work by Wearn and Richards provided the first experimental evidence that the glomerular filtrate was protein free and that the