Membrane Proteins

# Aquaporin Water Channels (Nobel Lecture)**

*Peter Agre**

**Keywords:**
aquaporins · medicinal chemistry · membrane proteins · Nobel lecture · protein structures

## From the Contents



Thank you very much. I am humbled, I am delighted; I am honored. It is every scientist's dream to give the Nobel Lecture in Stockholm. But I would not be honest if I did not tell you that I am having a little anxiety being on this platform. I have lectured a number of times in Sweden, and I thought I would share with you some events preceding a special lecture that I gave here a few years ago. Arriving at Arlanda Airport, I waited in line at the Passport Control behind a group of businessmen in suits with briefcases. I heard the first in line asked by the control officer to state the purpose of his visit to Sweden. When the man replied "business," the officer approved and stamped his passport. One at a time, each stepped forward and was asked the same thing; each answered "business" and was approved. Eventually it was my turn, and I was dressed in rumpled clothes after spending the night in the Economy Minus section of an SAS jetliner. The officer asked me the purpose of my visit, and I said "I am here to give the von Euler Lecture at Karolinska Institute." The officer immediately looked up, stared at me, and asked, "Are you nervous?" At that point I became intensely nervous and said "Yes, I am a little nervous." The officer looked up again and stated "Well, you should be!" So if the lecturers look a little nervous, the problem is at Arlanda.

## Introduction

I am going to talk about aquaporin water channels. We have studied these proteins for several years, and we now understand that they explain how water crosses biological membranes. Water is commonly regarded as the "solvent of life", since our bodies are 70% water. All other vertebrates, invertebrates, microbes, and plants are also primarily water. The organization of water within biological compartments is fundamental to life, and the aquaporins serve as the plumbing systems for cells. Aquaporins explain how our brains secrete and absorb spinal fluid, how we can generate aqueous humor within our eyes, how we can secrete tears, saliva, sweat, and bile, and how our kidneys can concentrate urine so effectively. These proteins are fundamental to mammalian physiology, but they are also very important in the lives of microorganisms and plants.

I wish to discuss the background in order to give credit to the individuals who were in this field long before we joined. With the recognition of the lipid bilayer as the plasma membranes of cells back in the 1920s, it was correctly proposed that water could move through the membrane simply by diffusing through the lipid bilayer. The current view is that the lipid bilayer has a finite permeability for water, but, in addition, a set of proteins exists that we now refer to as

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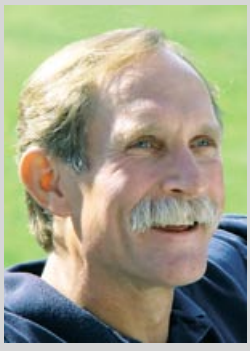
"aquaporins." Their existence was suggested by a group of pioneers in the water transport field, such as Arthur K. Solomon in Boston, Alan Finkelstein in New York, Robert Macey in Berkeley, Gheorghe Benga in Romania, Guillermo Whittembury in Venezuela, and Mario Parisi in Argentina, who preceded us by decades. They predicted by biophysical methods that water channels must exist in certain cell types with high water permeability such as renal tubules, salivary glands, and red cells (see the review by Finkelstein[1]).

The difference between diffusional and channel-mediated water permeability is fairly remote. Diffusion is a low capacity, bidirectional movement of water that occurs in all cell membranes, whereas the membranes of a subset of cells with aquaporin proteins have a very high capacity for permeation by water. This permeability is selective, since water ($H_2O$) passes through the membranes with almost no resistance, while the hydronium ion ($H_3O^+$) does not permeate the proteins. This distinction is essential to life. As I will discuss later, our kidneys reabsorb 99% of the water from the primary filtrate to prevent us from becoming dehydrated. If our kidneys reabsorbed water *and* acid, we would develop systemic acidosis.

The movement of water is directed by osmotic gradients, and so aquaporins are not pumps or exchangers. They form a simple pore that allows water to rapidly pass through membranes by osmosis, a phenomenon that we all learned about in middle school. There are also other differences between diffusion and channel-mediated water transport. No inhibitors are known for simple diffusion. In contrast, mercury compounds were discovered by Robert Macey to inhibit water transport in red blood cells, but water permeability was restored by treatment with reducing agents.[2] These observations led to the prediction that water channels must be proteins with sulfanyl groups and characteristically low Arrhenius activation energy.

A number of investigators attempted to identify the water channel molecule by using very logical approaches, but identification proved a very difficult problem. Water is ubiquitous and cannot be modified to include photoactivated side chains. Attempts at expression cloning were unsuccessful. Isotopic mercury compounds were used to label several membrane proteins: the anion-exchanger protein (band 3) by Solomon and a group of several proteins (band 4.5) by Benga. Nevertheless, none of the proteins were isolated, reconstituted, and shown to transport water (see the review in Ref. [3]).



*Peter Agre received his MD in 1974 from Johns Hopkins University (Baltimore, Maryland, USA). He was a post-doctoral researcher at Case Western Reserve University (1975–1978) and at the University of North Carolina at Chapel Hill (1980–1981). After research work in the group of V. Bennett (1981–1983) he became Assistant Professor at Johns Hopkins School of Medicine in 1984, and since 1993 he has been Professor of Biological Chemistry and Professor of Medicine. He was awarded the Nobel Prize for Chemistry in 2003 for his work on aquaporins.*

## The Discovery of AQP1

The field was essentially stuck, but following the well-known scientific approach known as "sheer blind luck", we stumbled upon the protein that is the answer to the question: Do water channels exist? Looking through our notebooks for the earliest studies that showed there was such a protein, I found an autoradiograph prepared by my first lab assistant Andy Asimos. I was at that time a hematologist, and we were studying the Rhesus (Rh) blood group antigens. We were attempting to raise antibodies in rabbits to the denatured partially purified Rh polypeptide. The rabbits vigorously produced antibodies that reacted with a polypeptide of approximately 30 kDa, and a series of higher-order oligomers and glycosylated proteins. We were very excited about this, but we failed to recognize initially that our antibody did not react with the core Rh polypeptide (32 kDa), as seen clearly by silver staining of sodium dodecyl sulfate polyacryamide electrophoresis gels (SDS-PAGE). Instead, our antibodies reacted only with a 28-kDa polypeptide that we had mistakenly believed to be a breakdown product of the larger Rh polypeptide. Thus, the 28 kDa was an unrelated protein that merely contaminated our Rh preparations.

A number of features about the 28-kDa protein created a large amount of interest. Postdoctoral fellow Brad Denker and laboratory assistant Barbara Smith isolated the protein by a simple approach based upon detergent solubility. Silver staining of the electrophoresis gels revealed a discrete band of 28 kDa in detergent-insoluble extracts. No one had seen this protein before, since it failed to stain with the conventional protein stains such as Coomassie blue. The protein was then purified in large amounts from the membranes of human red blood cells.[4,5]

The 28-kDa protein was strikingly abundant: With approximately 200000 copies per red blood cell, it was one of the major proteins in the membrane. In a way, this may be compared to the surprise one would have if, while driving along a road in northern Sweden, one came upon a town with 200000 people not on the map—it certainly got our attention. Moreover, the protein had features that suggested it was a tetrameric membrane-spanning protein and thus was a channel—but a channel for what? The purified protein also provided us with the N-terminal amino acid sequence that we used for cDNA cloning. Using our antibody, we looked at several other tissues and found the protein is also strikingly abundant in human kidney. We observed staining over the apical and basolateral membranes of proximal renal tubules and the descending thin limb of the loops of Henle, but we were still frustrated by our failure to recognize what the protein's function might be.

In science, one should use all available resources to solve difficult problems. One of our most powerful resources is the insight of our colleagues. I spoke to a dozen or more well-known biochemists and physiologists about this protein, but none could predict what its function might be. My clinical

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mentor, John C. Parker (1935–1993) at the University of North Carolina at Chapel Hill was the first to suggest to me that red blood cells and renal tubules were exceedingly permeable to water. He recommended that we consider a role in water transport through membranes. While John did not live to see our later studies, he did live to see our initial discovery and we celebrated together.

Gregory Preston, then a postdoctoral fellow in my research group, cloned the cDNA from an erythroid library.[6] The coding region corresponded to a 269 amino acid polypeptide, which was predicted by hydropathy analysis to have six bilayer-spanning domains. Interestingly, the amino terminal half (repeat 1) and the carboxy terminal half of the molecule (repeat 2) were genetically about 20% identical. The two loops B and E were more highly related to each other, and each contained the signature motif: asparagine, proline, alanine (NPA, Figure 1). By examining the genetics database we recognized several sequence-related DNAs from diverse sources: lenses of cow eyes, brains of fruit flies, bacteria, and plants. Nevertheless, none was functionally defined.

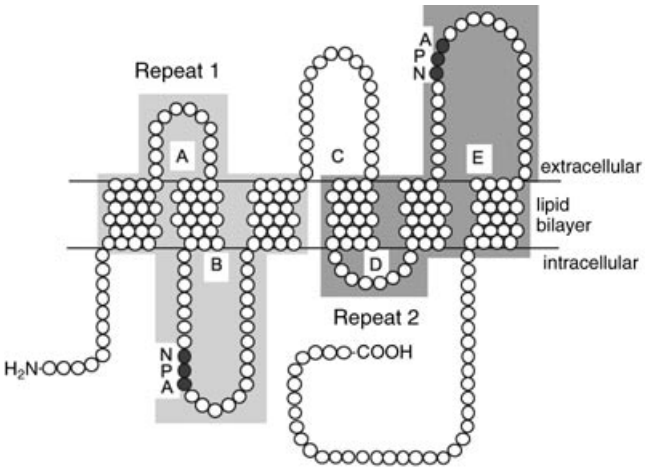


***Figure 1.*** Membrane orientation of AQP1 predicted from primary amino acid sequence. Two tandem repeat units of the protein each have three bilayer-spanning domains and are oriented 180° with respect to each other. The loops B and E each contain the conserved motif Asn-Pro-Ala (NPA).

These clues heightened our suspicion that the 28-kDa protein was a water transporter, so we tested for possible water-transport function in collaboration with our colleague Bill Guggino at Johns Hopkins University. We used oocytes from the frog *Xenopus laevis*. This convenient expression system was particularly useful, since frog oocytes have very low water permeability and survive even in fresh-water ponds. Control oocytes were injected with water alone; test oocytes were injected with 2 ng of cRNA encoding our protein. After three days of protein synthesis, the oocytes appeared essentially identical. Then we stressed the oocytes by transferring them to distilled water, and an amazing difference was immediately apparent: The control oocytes, having exceedingly low water permeability, failed to swell, while the test oocytes were highly permeable to water and exploded like popcorn (Figure 2).[7] This result produced much celebration in the laboratory; the celebrations continue still. The protein was christened "aquaporin" and is now officially designated "AQP1," the first functionally defined water-channel protein.[8]

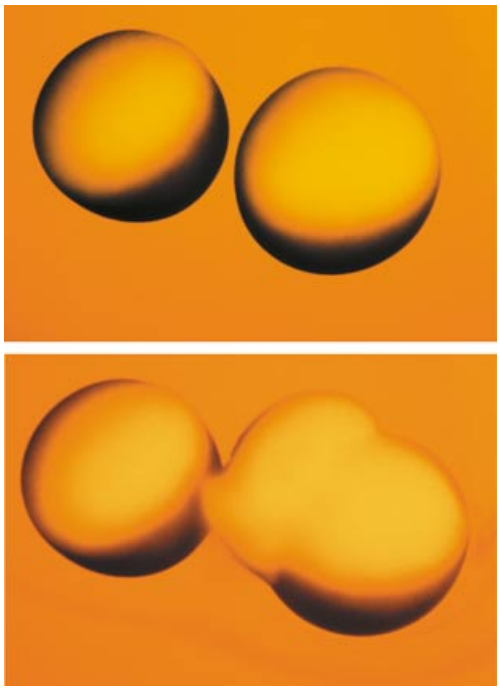

***Figure 2.*** Functional expression of AQP1 water channels in *Xenopus laevis* oocytes. The control oocyte (left) was injected with water, the test oocyte (right) was injected with AQP1-coding cRNA. The oocytes were transferred to hypotonic buffer. After 30 s (top) the AQP1 oocyte has begun to swell and after 3 min (bottom) it has exploded. Modified and reprinted with permission from Ref. [7].

We confirmed the function of this protein by studying the purified AQP1 reconstituted into synthetic lipid vesicles with diameters of about 0.1 microns prepared by our colleague Suresh Ambudkar at Johns Hopkins University.[9] These simple membrane vesicles were examined by freeze-fracture electron microscopy by our colleague Arvid Maunsbach, from the University of Aarhus. When lipid was reconstituted without protein, the membrane surfaces were smooth; however, membranes reconstituted with AQP1 contained many intramembraneous particles with diameters of 0.01 microns.[10] We tested the membranes for water permeability in collaboration with Mark Zeidel at Harvard Medical School. The simple liposomes shrank on transfer to hypertonic buffer by using stopped-flow techniques, and reached equilibrium in about one half second; this is believed to represent the baseline water permeability. When membranes reconstituted with AQP1 were examined, the shrinking occurred much more rapidly, with equilibrium reached in about 20 ms. The channel-mediated flow of water was confirmed, since it was inhibited with mercury compounds. We calculated the Arrhenius activation energy ($< 5$ kcal $mol^{-1}$), and determined the unit permeability to be about $3\times$

  

$10^9$ water molecules per subunit per second. Importantly, we attempted to measure proton permeation of AQP1, but despite massive water permeability, acid permeation was not detected. These studies verified that we had, in fact, isolated the long-sought water-channel protein.

## *Structure of AQP1*

Subsequent efforts were devoted to identifying the binding site for the mercury inhibitors predicted by the studies of Macey. Mercury compounds react with free sulfanyl groups in cysteine. Four cysteine residues are found in the AQP1 polypeptide, but only the residue in loop E (Cys-189 proximal to the second NPA motif) is inhibited by mercury compounds. We altered the AQP1 sequence by site-directed mutagenesis and expressed the recombinants in oocytes for water permeability studies. Mutation of this residue to serine (Cys-189-Ser) resulted in full water permeability without inhibition by mercury compounds. When we then replaced the alanine residue in the corresponding position of loop B with a cysteine residue (Ala-73-Cys), the protein exhibited water permeability that was sensitive to the presence of mercury compounds.[11] Substitutions elsewhere in the AQP1 failed to produce this behavior. This result suggested to us that loops B and E in opposite parts of the molecule must somehow form the aqueous pore. The model that we concocted turned out to be schematically correct and was termed "the hourglass"—an ancient timepiece that allows sand to run from the upper chamber to lower chamber; if inverted, the sand will flow in the opposite direction.

Six bilayer-spanning domains were predicted to surround a central domain containing loop B, which dips into the membrane from the cytoplasmic surface, and loop E, which dips into the membrane from the extracellular surface (Figure 3). The overlap of loops B and E was predicted to form a single aqueous pore down through the center of the molecule with the NPA motifs juxtaposed and the binding site of the mercurial inhibitors alongside.[12] The AQP1 protein is a tetramer with a central pore in each subunit. Thus, AQP1 is structurally unlike ion-channel proteins where four subunits surround a single central pore.

We then sought to establish the high-resolution structure of AQP1 in collaboration with Andreas Engel and his research group at the Biozentrum in Basel. We were later joined by Yoshinori Fujiyoshi and his research group from Kyoto University. AQP1 protein from human red blood cells was purified by Barb Smith in our laboratory; Andreas's student Tom Walz reconstituted it into synthetic membranes at very high protein concentrations. Under these conditions the AQP1 protein forms remarkably symmetrical arrays that are referred to as membrane crystals. By measuring the water permeability, we confirmed that the function was 100% retained, thus giving us confidence that the structure we deduced would be the biologically relevant structure.[13]

By using the techniques of cryoelectron microscopy and atomic force microscopy, our colleagues in Basel and Kyoto undertook studies that provided an electron-density map of human AQP1 at a resolution of 3.8 Å. Models were developed using constraints established by our site-directed mutagenesis studies.[14] When a single AQP1 subunit is viewed in cross-section in the middle of the membrane bilayer, a single aqueous pore is visible with hydrophobic residues lining most

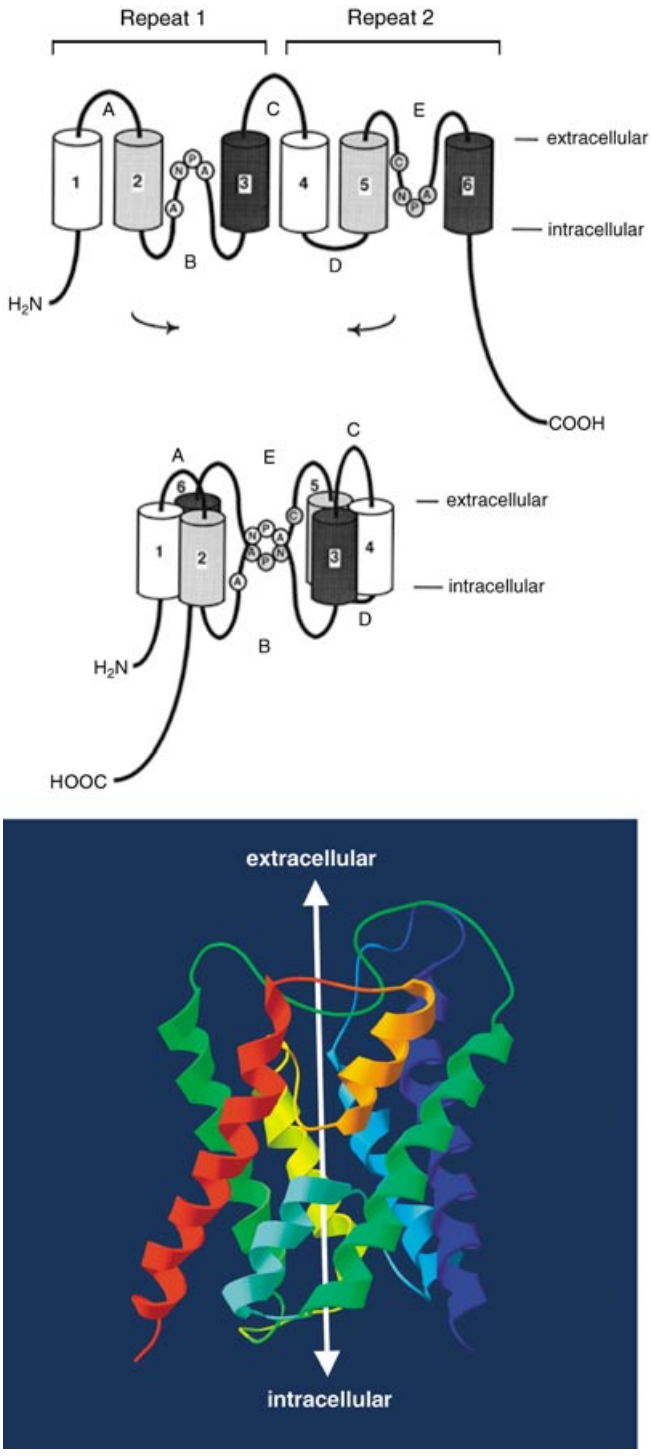


***Figure 3.*** Hourglass model for the membrane topology of the AQP1 subunit. Top: Schematic representation of the folding of loops B and E; the loops overlap within the lipid bilayer to form a single aqueous pathway. Bottom: Ribbon model of the three-dimensional structure of the AQP1 subunit. Modified and reprinted with permission from Refs. [12] and [19].

  

of the circumference of the wall, but the two highly conserved asparagine residues of the NPA motifs are aligned on the other wall. When viewed in longitudinal section, a narrow aqueous pore is visible with the two highly conserved asparagine residues.

We faced the question of how a simple pore with no moving parts could allow rapid transit of water with no movement of protons. Exciting contributions by X-ray crystallographers in the groups of Robert Stroud at UCSF and Bing Jap at Lawrence Berkeley Laboratory solved the atomic structures of the glycerol facilitator GlpF from *E. coli*,[15] and AQP1 from bovine red blood cells[16] at 2.2-Å resolution. These observations enabled molecular dynamics simulations to be performed by Bert de Groot and Helmut Grubmüller at the University of Göttingen[17] and the group of Klaus Schulten at University of Illinois at Urbana-Champaign.[18] We now understand how this protein facilitates the movement of water but not protons. If water were simply $H_2O$, it would be a gas (such as in steam), but water in a bulk solution exists as $(H_2O)_n$, with hydrogen-bonding interactions between adjacent water molecules causing it to be a liquid. If water existed as a column of aligned water molecules in contact with each other, protons would rapidly jump along by a mechanism known as the Grotthus effect ("proton wire").

The hourglass structure of the AQP1 molecule has an extracellular vestibule and an internal vestibule where water is in bulk solution. These vestibules are separated by a distance of approximately 20 Å, and linked through a channel which is so narrow that water moves through in single file while protons are reflected. Near the top of this bridge the channel reaches its narrowest constriction of 2.8 Å. Thus, the pore is just large enough to accommodate a single water molecule. At this site, the side chain of a perfectly conserved arginine residue following the NPA motif in loop E forms a fixed positive charge, and a conserved histidine residue on the other wall forms a partial positive charge; together they serve to repel protons. Further along, another barrier to protons exists where a single water molecule will transiently undergo a transient dipole reorientation as it simultaneously forms hydrogen bonds with the side chains of the two asparagine residues in the juxtaposed NPA motifs. In addition, the nonbilayer-spanning α-helices in the distal ends of loops B and E contribute partial positive charges that further serve to block proton conduction (Figure 4 left; see also the review by Kozono et al.[19]). The right-hand side of Figure 4 shows how mercury compounds inhibit water flow through AQP1. The side chain of Cys-189 lies along the pore, so if it is blocked by a mercury compound, the channel will be occluded.

## The Aquaporin and Aquaglyceroporin Protein Family

While we were pursuing studies of AQP1, several other research groups from around the world became interested in

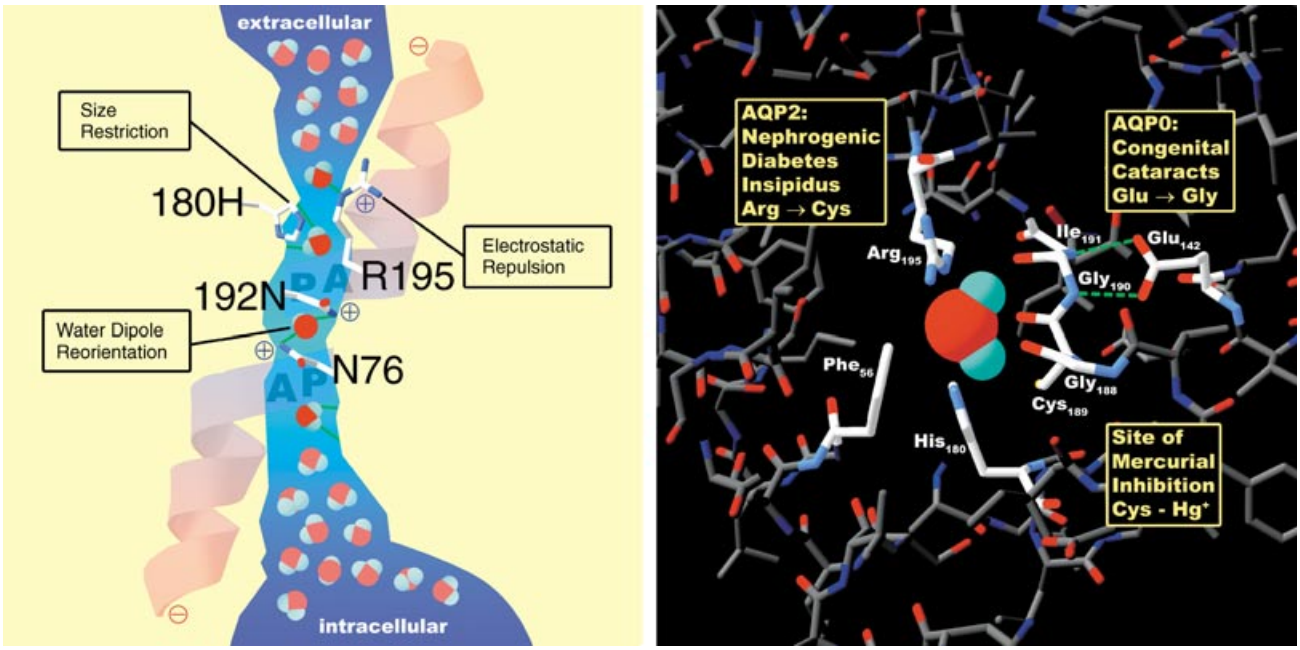


***Figure 4.*** Schematic representation of the selective flow of water through the AQP1 subunit and residues involved in human disease. Left: The sagittal cross-section of AQP1 reveals bulk water in the extracellular and intracellular vestibules of the hourglass structure. These vestibules are separated by a 20-Å channel through which water passes in a single file with transient interactions with pore-lining residues that prevent the formation of hydrogen bonds between the water molecules. Two structural elements are believed to prevent permeation by protons ($H_3O^+$): 1) electrostatic repulsion is created by a fixed positive charge from the pore-lining arginine residue (R195) at a 2.8-Å narrowing in the channel; 2) reorientation of the water dipole occurs from the simultaneous hydrogen bonding of water molecule with the side chains of two asparagine residues in NPA motifs (N192 and N76). Two partial positive charges at the center of the channel result from the orientation of two nonmembrane-spanning α-helices distal to the NPA motifs. Right: Space-filling representation of a water molecule at the narrowest region of a pore (cross-section). The binding site of mercury inhibitors at cysteine residue Cys189 in AQP1; the AQP2 mutation in nephrogenic diabetes insipidus (Arg→Cys); and AQP0 mutation in congenital cataracts (Glu→Gly) are highlighted. Reprinted with permission from Ref. [19].

  

what is now known to be a large family of related proteins. The combined efforts of these labs have led to the molecular identification of 12 mammalian aquaporin homologues, and several hundred related proteins have been recognized in other vertebrates as well as invertebrates, plants, and unicellular microorganisms. The mammalian homologues may be loosely clustered into two subsets (Figure 5). The first is referred to as "classical aquaporins", since they were initially considered to be exclusive water pores. The second is referred to as "aquaglyceroporins", since they are permeated by water and glycerol. Interestingly, *E. coli* has one member of each, AqpZ[20] and GlpF, isolated. Together, the mammalian aquaporins and aquaglyceroporins are now known to contribute to multiple physiological processes that occur during our daily lives.

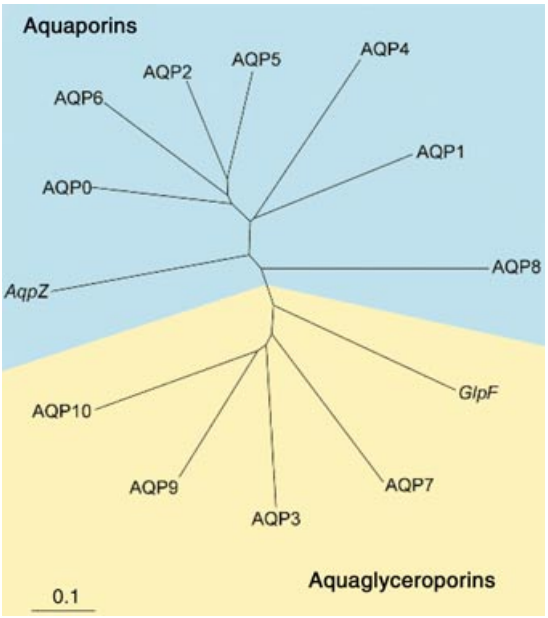


***Figure 5.*** The human aquaporin gene family contains two subsets: Homologues freely permeated by water (classical aquaporins, blue) or water and glycerol (aquaglyceroporins, yellow). *E. coli* has one aquaporin (AqpZ) and one aquaglyceroporin (GlpF). The scale bar corresponds to the genetic distance between homologues. Reprinted with permission from Ref. [65].

## *AQP1 Proteins in Kidneys*

We were very fortunate to collaborate with Søren Nielsen from the University of Aarhus, a superb microscopist with particular expertise in kidney physiology. Each of our two kidneys contains approximately one million nephron units working in concert to eliminate toxic wastes and provide the appropriate pH value and ionic composition of our circulating blood. Every day, approximately 180 L of plasma are filtered through the capillary tufts in the glomeruli. Water and certain solutes in this primary filtrate are then reabsorbed as the fluid makes its way through the tubules. The proximal convoluted tubules and descending thin limbs of the loop of Henle exhibit constitutively high water permeability and are responsible for the countercurrent mechanism for the concentration of urea in the kidneys. This coincides exactly with the sites where AQP1 is expressed. The ascending segments are permeated by certain ions and small molecules but lack the AQP1 protein and exhibit low water permeability. Multiple nephron units empty into collecting ducts which exhibit low water permeability in the unstimulated state. Collecting ducts are known to become highly permeable to water when stimulated by the antidiuretic hormone vasopressin, which is released from the brain in response to dehydration.

Immunohistological analysis of rat kidney by Søren Nielson revealed an extremely strong labeling of the proximal tubules and descending thin limbs with our affinity purified anti-AQP1.[21] The apical brush border stains intensely; our analyses indicate that AQP1 comprises about 4% of the total brush border membranes. Immunogold electron microscopy showed that AQP1 is largely present at the apical brush border membrane surface, where water reabsorption is known to occur, but the protein is present at a minimal level within the cell body (Figure 6 left).

Thus, it became clear that AQP1 is not actively trafficking from intracellular sites to the plasma membrane and back to intracellular sites. AQP1 is also abundant in the basolateral membranes of these epithelial cells. Thus, water is reabsorbed in a transcellular route from the tubular lumen through the apical membrane of the tubular epithelium and out through the basolateral membrane into the interstitium where it is absorbed into capillaries and venules that are also rich in AQP1 protein (Figure 6 right). In normal circumstances, water does not flow between cells, since tight junctions form a barrier. The direction of the water flow through these

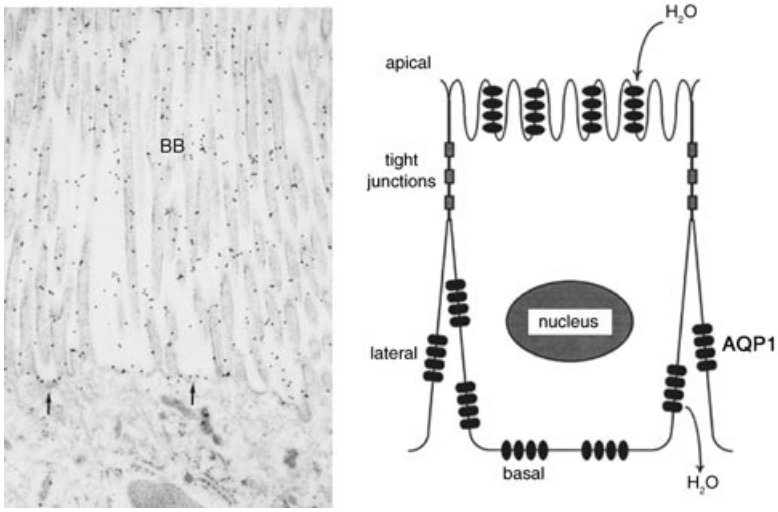


***Figure 6.*** Subcellular localization of AQP1 in proximal tubules from rat kidney. Left: Anti-AQP1 labeling studies using immunogold electron microscopy reveals labeling of apical brush border (BB) but not cell body (25000×). Right: Schematic representation of the transcellular passage of water: entry through AQP1 in the apical brush border and exit through AQP1 in basolateral membranes. Reprinted with permission from Ref. [21].

  

epithelia, from the primary filtrate back into the vascular space, is determined by small, standing osmotic gradients created by the solute transporters, and water follows passively.

## AQP1-null Phenotypes

Chulso Moon, one of our students in the Graduate Program in Human Genetics at Johns Hopkins University, was studying the structural gene encoding human AQP1 which we located on the short arm of human chromosome seven by fluorescence in situ hybridization (FISH).[22] We looked in Victor McKusick's atlas of human genetic linkages for similar gene sequences, and our friend Colvin Redman at the New York Blood Center pointed out that this location coincides with the site of an interesting blood group system: the Colton (Co) antigens. Being a hematologist, I am very much interested in blood group antigens. While hundreds of blood group antigens are known, they represent multiple polymorphisms in approximately 25 different blood group systems, with each system being a different genetic locus. So the coincidental localization of the *AQP1* gene and a human blood group antigen was a very strong clue that they correspond to the same molecule. The Co blood group antigens are not of large clinical significance, since most of us have the same Co blood type—approximately 90% of us are homozygous for $Co^a$, almost 10% are heterozygotes carrying both $Co^a$ and $Co^b$, and only 0.3% are homozygous for $Co^b$. In collaboration with investigators from the International Blood Group Referencing Laboratory in Bristol, UK, we found the Co antigens correspond to an alanine–valine polymorphism at residue 45 which resides in an extracellular location of the AQP1 protein.[23]

Surprisingly the blood group referencing laboratories have identified only a handful of individuals in the entire world who lack both Co antigens. We turned our attention to the Co-null individuals, and, with the help of John Moulds and Jean Pierre Cartron, we obtained DNA, blood, and urine from three different Co-null kindreds. The probands were all women who apparently became sensitized to the Co antigens on the red cells of their babies during childbirth. We hypothesized that these individuals carried mutations in their genes encoding AQP1. We found that the probands from each kindred were homozygous for a unique disruption of the *AQP1* gene:[24] They do not express AQP1 in red blood cells, renal tubule cells, or presumably in choroid plexus, ciliary epithelium, or any other tissues where the protein is normally found—hence they are termed AQP1-null. The probands, however, did not show any clinical illness. Their unique blood types make it impossible for them to receive transfusions from any other individual, so each has cryopreserved blood in local blood banks for emergencies or if a transfusion is needed during surgery.

Their lack of clinical symptoms suggested that AQP1 is either not important in humans or is important only in certain physiological states or clinical settings. Studies of mice bearing targeted disruption of the gene encoding AQP1 by Alan Verkman at UCSF and collaborating scientists at NIH indicated that AQP1 is important for renal concentration.[25,26] Our colleague Landon King at Johns Hopkins University designed a series of clinical evaluations to probe for the importance of the human AQP1 protein. Two AQP1-null individuals were studied during inpatient evaluations at the General Clinical Research Center at Johns Hopkins Hospital. Water permeability is clearly reduced in AQP1-null red cells, but all baseline studies were found to be normal. Then Landon performed a simple test: he thirsted the patients. Every night during our sleep we become a little dehydrated; upon arising in the morning that first taste of orange juice is so delicious. To protect against dehydration, our kidneys respond by maximally concentrating our urine, and all normal individuals concentrate to approximately 1000 mosmol $L^{-1}$. The AQP1-null individuals were found to have urine concentrations reaching no higher than 450 mosmol $L^{-1}$ after overnight thirsting, and prolonged thirsting to 24 h followed by administration of the antidiuretic hormone vasopressin and infusion of hypertonic sodium chloride yielded no further response.[27] In modern life, with free access to fluids and air conditioning, the need to concentrate above 450 mosmol $L^{-1}$ is not essential; however, the AQP1-null individuals would be at a profound disadvantage if they were to reside in an environment with restricted access to fluids. Certainly during human evolution, our progenitors did not always have free access to water. The patients are described as having a mild form of nephrogenic diabetes insipidus that is usually subclinical but physiologically distinct.

Søren Nielsen also established that AQP1 protein is extremely abundant in human capillaries in both the luminal and abluminal endothelial membranes.[28,29] Landon King in collaboration with Robert Brown measured the capillary fluid permeability in AQP1-null humans.[30] By using a technique developed by Wayne Mitzner and Elias Zerhouni at Johns Hopkins University that employed the high-resolution computerized tomography of lung, they performed scans at a baseline level. By focusing upon small bronchioles of 2–3-mm diameter, the cross-sectional areas of the adjacent venules were measured and the wall thicknesses determined. The scans were repeated after rapid infusion of three liters of saline. All normal individuals and AQP1-null individuals exhibited comparable evidence of vascular engorgement following saline infusions. We then looked at the wall thickness of the small breathing tubules and found that all normal individuals developed an increase in the wall thickness, which represents a release of fluid from the venules and accumulation in the soft tissues surrounding the bronchioles—a situation referred to by clinicians as incipient pulmonary edema. The interstitial edema appears in the peribronchiolar soft tissues in all normal individuals, but the AQP1-null individuals are resistant to this, thus indicating to us that they have a very significant reduction in vascular water permeability. Whether this may be beneficial or deleterious will depend on the clinical setting. At the time of birth, lungs turn from a secretory organ to an absorptive organ. We hypothesize that the inability of newborn infants to move fluid from the pulmonary intersitium into the vascular space may be very harmful, possibly explaining why the AQP1-null state is such a rare phenotype.

  

## *Other Aquaporins—AQP2 in the Collecting Ducts*

Within weeks of our original studies, other research groups began to search for other aquaporins. Particular interest in renal collecting ducts led Sei Sasaki and his colleagues at Tokyo Medical and Dental University to the isolation of a cDNA encoding AQP2.[31] Søren Nielsen et al. established that AQP2 is primarily expressed in renal collecting ducts where regulated water permeability is known to occur.[32] Although my research group was not involved in the studies of AQP2, I wish to share exciting information published by several other investigators because I believe this has very large potential clinical significance.

In a definitive study, Søren Nielsen, Mark Knepper, and co-workers from NIH looked at AQP2 in collecting ducts isolated from rats.[33] The ducts were perfused while water permeability was measured. Some of the specimens were then sectioned and stained with anti-AQP2, and they found by immunogold electron microscopy that very little of the AQP2 protein was found in the apical plasma membrane of collecting duct principal cells, but most of the protein appeared to be in membranes of intracellular vesicles (Figure 7, upper left). When the isolated perfused tubules were pulsed with physiological concentrations of vasopressin (the physiological antidiuretic hormone released from the brain), the water permeability was increased approximately fivefold, which coincided with the expected physiological increase. Immunogold electron microscopy studies showed that the AQP2 protein had relocated to the apical plasma membrane (Figure 7, lower left). When vasopressin was removed, the AQP2 protein was re-internalized and the collecting duct water permeability decreased.

These observations closely reflect the known variations in the water permeability if the renal collecting duct occurring in our daily lives. On Friday afternoons, our students celebrate the end of the week at the departmental beer party (TGIF = Thank God it's Friday). A student drinking two liters of beer has excess fluid in his system and the alcohol inhibits release of vasopressin from brain. This causes AQP2 to remain in intracellular vesicles, so the apical membranes of renal collecting duct principal cells are not able to reabsorb water from the filtrate (the diuretic state). The first place the student visits following the TGIF party is the toilet where he empties his bladder of a large volume of dilute urine. The next morning, the student lies in bed in a moderately dehydrated state with high vasopressin levels in his circulation. This causes AQP2 to relocalize to the apical plasma membranes, facilitating maximal reabsorption of water from the urine. Thus, when he arises, his urine is maximally concentrated (the antidiuretic state). These cycles may be repeated multiple times even within a single day.

In summary, vasopressin binds to V2 receptors at the basolateral membrane of the renal collecting duct causing activation of a G-coupled adenylylcyclase cascade that results in phosphorylation of residue 256 on the C-terminus of AQP2 by protein kinase A. The protein is then inserted into the apical plasma membrane, as established by Nielsen and Knepper, the research groups of Deen in Nijmegen, Dennis Brown in Boston, Sasaki in Tokyo, Anita Aperia in Stockholm, and others. Water in the primary urine enters the apical membrane through AQP2 and exits from the basolateral membranes through other aquaporins (AQP3 or AQP4) following a transcellular pathway from the lumen into the vascular space (see the review by Nielsen et al.[34])

Inherited defects in AQP2 cause very rare but severe clinical problems, as found by Carel van Os, Peter Deen, and their colleagues in Nijmegen, The Netherlands.[35] These patients are profoundly affected, they make up to 20 L of urine per day in some cases. Acquired defects in the level of AQP2 expression are commonly found in clinical medicine. When AQP2 levels are too high, fluid retention occurs (as shown by the Nielsen and Knepper research group as well as by Robert Schrier and his colleagues at the University of Colorado). Presumably this is happening in patients sustaining myocardial infarctions; such patients often suffer fluid retention which causes inadequate pulmonary function. Similarly, some women during pregnancy experience pathological retention of fluid, which causes hypertension and neurological dysfunction. The opposite situation results when AQP2 levels are too low, resulting in inadequate concentration of urine. This result is found after reversal of urinary obstruction (by Jørgen Frøkiaer in Aarhus) and in patients with nocturnal enuresis (by

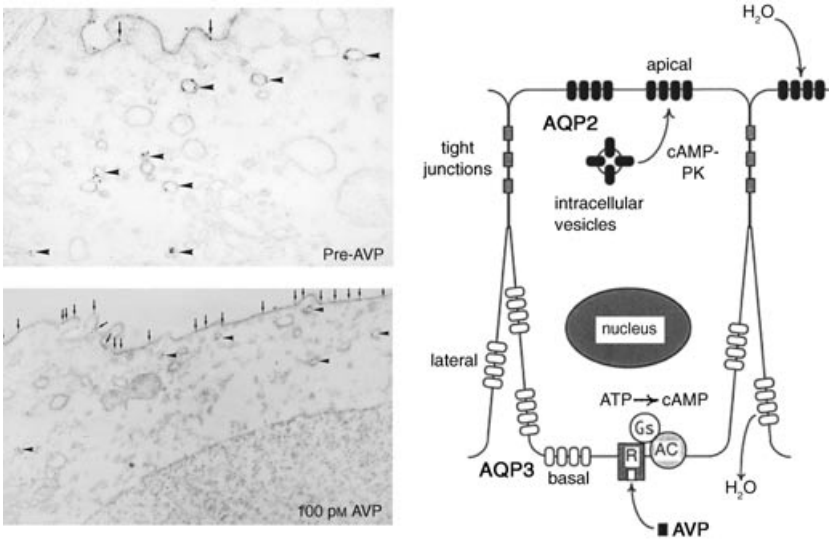


***Figure 7.*** Subcellular localization of AQP2 in collecting duct principal cells from rat kidney. Left: Anti-AQP2-labeled immunogold electron microscopy of isolated collecting duct from rat kidney. Before vasopressin (AVP) stimulation, most labeling occurs at the intracellular vesicles (top, 25000×). After stimulation with 100 pM AVP, most labeling appears at the apical membrane (bottom, 15000×). Right: Schematic representation of the AVP-dependent exocytosis of AQP2 to the apical membrane of the principal cell: entry through AQP2 in apical membrane, exit through AQP3 (or AQP4) in the basolateral membranes. Modified and reprinted with permission from Refs. [33a] and [33b].

Ivana Valenti in Bari). Thus, AQP2 is very important in clinical medicine (see the review by Nielsen et al.[33])

### AQP6 in Acid Secretory Cells

At this point we thought we knew a lot about aquaporins, but then we were surprised. AQP6, a protein genetically closest to AQP2, was found to be a very poor water channel and is never present in the plasma membrane. Double-label immungold electron microscopy studies by Søren Nielsen and Tae-Hwan Kwon showed that AQP6 colocalizes with $H^+$-ATPase in intracellular vesicles of α-intercalated cells in the renal collecting duct. Upon stimulation of acid secretion, $H^+$-ATPase becomes exocytosed to the plasma membrane, but AQP6 is fully retained within the cell. A second surprise was that AQP6 is a very good anion channel with particularly high permeation by nitrate when activated by low pH values.[36] These cells control acid secretion, so while AQP6 is clearly a genetic member of the aquaporin family, it apparently has a very different physiological function, as proposed by our colleague Masato Yasui at Johns Hopkins University.

### AQPo in Lenses

Another member of the aquaporin family is AQP0, which is expressed only in fiber cells of lenses. First recognized more than 30 years ago and termed MIP (major intrinsic protein), the physiological function was unknown. AQP0 has a fairly low water permeability, but the protein may have a second function as a cell-to-cell adhesion molecule, as proposed by Andreas Engel and co-workers.

Shomi Bhattacharya, from Moorefield's Eye Hospital in London, identified two large kindreds with congenital cataracts of dominant inheritance with complete penetrance. While cataracts are fairly common among elderly individuals, they are very rare in small children. Each family bore a missense mutation in a structurally important residue, and the physical nature of the opacities was quite different.[37] Members of the family with an arginine replacing an essential threonine residue (Thr-138-Arg) have numerous opacities throughout the lens. Members of the second family with a glycine replacing an essential glutamate residue (Glu-134-Gly) have a single lamellar opacity in the center of the lens. This distribution corresponds to the perimeter of the lens at the time of birth, thus indicating that the mutation is deleterious only at birth.[38] This example illustrates how human biology is so much more complicated than represented by simple gene disruption studies performed on mice. Lens fiber cells survive for decades, so it is plausible that subtle polymorphisms in the AQP0 protein may be a risk factor for the development of cataracts as individuals grow older.

### AQP4 in the Brain

AQP4 is expressed in multiple tissues, including the brain.[39] Unlike peripheral capillaries that are quite leaky, capillaries in the brain are known to be much tighter because of the astroglial endfeet surrounding them in the brain. In studies undertaken with Ole Petter Ottersen and his research group at the University of Oslo, and including Søren Nielsen, the protein was shown by immunogold electron microscopy to reside at the blood–brain barrier, the site where vascular space and brain parenchyma are juxtaposed.[40,41] Brain capillaries are surrounded by a basement membrane that is surrounded by astroglial endfeet. The AQP4 resides almost entirely in the endfeet membranes, directly in contact with the basement membrane and is virtually absent in other astroglial membranes (Figure 8a, b).

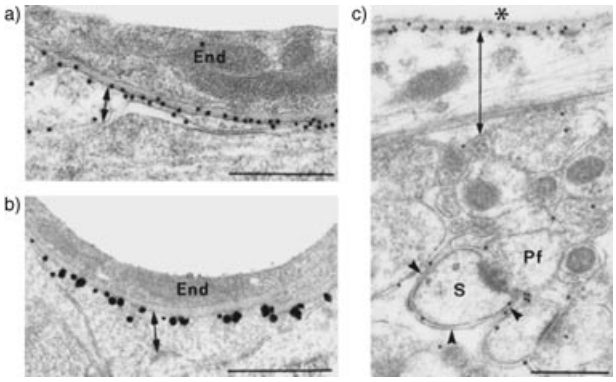


***Figure 8.*** Polarized expression of AQP4 in rat brain visualized by anti-AQP4 immunogold electron microscopy. a, b) AQP4 in perivascular membranes of astroglial endfeet but not in membranes facing the neuropil. c) Labeling of pia on the brain surface. Scale bars: 0.5 μm. End = endothelium, Pf = parallel fibers, S = spike of a Purkinje cell, ✱ = pia surface. Reprinted with permission from Ref. [39].

Similarly, at the surface of the brain, AQP4 is abundant at the brain surface where liquid and brain are juxtaposed (Figure 8c). These locations strongly suggested that AQP4 would be important in the movement of water from blood or cerebrospinal fluid into the brain as well as in the reverse direction. AQP4 is extremely heavily expressed at these locations and generate microcrystalline forms referred to as "square arrays", with the size of the assembly determined by the N-terminus of AQP4.[42] Other workers, including Antonio Frigeri in Bari, also documented that movement of water in brain and skeletal muscle may be of pathophysiological significance.[43]

Our student John Neely recognized a protein-binding motif in the C-terminus of AQP4 that predicted that it would associate with dystrophin-associated proteins. In collaboration with Marv Adams and Stan Froehner in Seattle as well as with our colleagues in Oslo, we evaluated the localization of AQP4 in mice bearing targeted disruption in α-syntrophin (syn), a member of the dystrophin-associated proteins.[44] AQP4 was found by immunogold electron microscopy to be grossly mislocalized in syn-null animals. Interestingly, when stressed with brain injury resulting from middle cerebral artery occlusion, the control animals did much worse, having significantly larger areas of infarction and edema, while the syn-null animals did much better.[45] These studies suggest that

  

if AQP4 could be selectively inhibited, patients sustaining brain injuries might be protected from some of the tragic consequences of brain swelling. Protection against hyponatremia-induced brain swelling was observed by Søren Nielsen and co-workers.[46] Geoff Manley and colleagues independently established the same conclusions with AQP4-null mice.[47]

## AQP5 in Secretory Glands

AQP5 resides in the apical membrane of acini in salivary, lacrimal, sweat, and other secretory glands.[48,49] This is the last membrane water crosses during the generation of saliva, tears, and other secretions. It is known that these glands facilitate the release of large amounts of fluid. For example, any individual fasted for several hours will secrete massive amounts of saliva when he senses food is available.

AQP5 may be important in certain clinical disorders. In collaboration with Kazuo Tsubota from Tokyo and Chris Delporte from Brussels, we examined AQP5 distribution in gland biopsies from a small series of patients with Sjögren's Syndrome. This disorder affects women at midlife and causes dry eyes and a dry mouth, although the precise biological cause of the disorder is unknown. The normal apical staining is lost with our anti-AQP5 immunohistochemical labeling, and the protein appears to be mislocalized throughout the cytoplasm in the Sjögren's biopsies.[50,51] Other investigators led by Roland Jonsson in Bergen did not find AQP5 mislocalization in a larger series of Sjögren's patients; however, they noted decreased expression of AQP1 in the myoepithelia surrounding glandular acini.[52] Thus, while Sjögren's Syndrome may be heterogeneous, aquaporins have been implicated in these two series of patients.

AQP5 is also abundant in sweat glands. In another collaborative study with Søren Nielsen and his research group, the qualitative analysis of sweating was compared in wild-type mice and mice bearing targeted disruption of the the gene encoding AQP5 (prepared by Carissa Krane and Anil Menon at the University of Cincinnati). While the morphology of the sweat glands was preserved, the release of amylase in sweat after pilocarpine injection was found to be greatly diminished, thus indicating that AQP5 must be important for the ability to sweat.[53]

## Aquaglyceroporins AQP7 and AQP9

AQP7 is expressed in adipocytes. During fasting, energy stored as triglycerides is provided by release of fatty acids, exported by special fatty acid transporters, and glycerol, exported through AQP7. The preferred gluconeogenic substrate, glycerol, passes through AQP9 in the hepatocyte plasma membranes. Glycerol is converted into glucose within the heptacytes. In the fasted state, AQP9 expression is greatly increased, as demonstrated in the work of Jennifer Carbrey and Dan Gorelick in our research group. Multiple research groups have reached the same conclusion that these two proteins (AQP7 and AQP9) form an axis for energy transfer, together maintaining blood-glucose levels during starvation.[54,55]

AQP7 and AQP9 have a very curious capacity to transport certain heavy metals, a discovery made by Barry Rosen and his colleagues at Wayne State University.[56] Measurement of arsenic trioxide uptake was performed with yeast expressing AQP7 or AQP9 as well as in oocytes expressing the proteins. Arsenic trioxide is uncharged at neutral pH values and is very toxic. Clinical features of greatest concern in arsenic poisoning include hepatocellular damage and hepatocellular carcinoma. The World Health Organization has established that an alarming epidemic of arsenic poisoning is now occurring in the Ganges Delta because of arsenic contamination of the groundwater. Tube wells were installed because the surface water is infested with *Vibrio cholera*, but heavy-metal determinations were apparently not performed. While our work explains why these unfortunate people are vulnerable to hepatocellular damage, it does not provide a useful antidote. Prevention of this poisoning requires access to pure drinking water, a basic right deserved by all persons on Earth.

## Nonhuman Aquaporins

Aquaporins are also important in plants. *Arabidopsis thaliana*, a relative of the mustard plant, is frequently studied by plant physiologists (also known as "the green people"). Ralf Kaldenhoff at the University of Würzburg demonstrated this dramatically by comparing the gross morphology of a parental *Arabidopsis* to a plant modified to express the rootlet aquaporin group PIP1b at only 20% of the normal levels. Both plants exhibit similar foliage, but to maintain the same stem turgor, the modified plant sends out more greatly arborized rootlets (Figure 9).[57] Thus, the plants are keenly aware of water balance. *Arabidopsis thaliana* has at least 35 different aquaporin genes, and these are presumed to participate in different physiological processes, including photosynthesis (in which carbon dioxide passes through leaf aquaporins)[58] and restriction of rootlet water uptake regulated by low pH values.[59] These observations may be relevant

***Figure 9.*** Rootsystem of *Arabidopsis thaliana*. Reprinted with permission from Ref. [57].

  

to human aquaporins, since similar observations have been reported for AQP1[60] and AQP3.[61]

Aquaporins are also expressed in all forms of microorganisms. *Escherichia coli* has two: AqpZ, a classic aquaporin, and GlpF, an aquaglyceroporin. The structures of the narrowest sections of the channels are somewhat different: narrower and more hydrophilic in aquaporins than in aquaglyceroporins. Archaea, the third kingdom of life, also carry a gene that encodes AqpM, which is homologous to aquaporins and aquaglyceroporins. AqpM may not exist for water transport but may facilitate hydrogen sulfide transport.[62] Whatever the function, their presence in all three kingdoms of life reinforces the fundamental importance of this family of proteins. *Sacharomyces cerevisiae* have two aquaporin genes and two aquaglyceroporin genes. Exciting studies by Stefan Hohmann at the University of Göteborg revealed interesting functions: yeast aquaporins confer resistance to freezing, whereas the aquaglyceroporin FPS1 protects the organisms against hypoosmotic shock.[63]

## Conclusion

The discovery of the aquaporins has provided the explanation for the selective transport of water across the plasma membranes of cells without allowing protons to also cross the membrane. Some homologues have special permeation properties for glycerol, nitrate, or arsenic trioxide. The structural models of aquaporins provide remarkable insight into the biophysical functions. Aquaporins have been implicated in numerous clinical disorders and are clearly involved in some forms of renal vascular diseases, including nephrogenic diabetes insipidus. Roles in defense against thermal stress are expected. Last summer during the heat wave in France, 14000 people died when the temperature reached 40 °C. Many of these individuals were older, and it is likely that they manifested age-dependent diminution of physiological processes involving aquaporins (concentration of urine, sweating, and thirst mechanisms). While these deficiencies may be unimportant during normal life, they may cause severe problems when individuals are thermally stressed. Aquaporins have also been associated with problems of brain edema and loss of vision. Aquaglyceroporins are involved in the defense against starvation. These proteins are expressed throughout the natural world.

The advice given to his students by Santiago Ramón y Cajal, the father of neuroscience, "In summary, there are no small problems. Problems that appear small are large problems that are not understood"[64] also applies here: Certainly the issue of water permeation of membranes was initially assumed to be a small problem related to diffusion. Instead it has turned out to be a complex problem with large physiological and pathophysiological significance.

*I thank the past and present members of my laboratory and the many individuals with whom we have collaborated in these studies. I particularly wish to thank my former chairmen, Victor McKusick, Department of Medicine, and M. Daniel Lane, Department of Biological Chemistry, for hiring me onto their faculty. I dedicate this lecture to the memory of John C. Parker, a friend and generous mentor.*

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