



THE STORY OF THE DISCOVERY OF AQUAPORINS: CONVERGENT EVOLUTION OF IDEAS – BUT WHO GOT THERE FIRST?

P. W. KUCHEL[✉]

School of Molecular and Microbial Biosciences,
Building G08, University of Sydney, NSW 2006, Australia
Tel.: +61 2 9351 3709; Fax: +61 2 9351 4726; E-mail: p.kuchel@mmb.usyd.edu.au

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Abstract – A critical analysis of the discovery of the first water channel protein (later called aquaporin 1) has been performed. In 1986 Benga's group in Cluj-Napoca, Romania, published in *Biochemistry*, a US-based journal, the results of experiments that provided the first visual and tangible evidence that the very rapid water exchange that occurs through the membranes of the human red blood cell (RBC) is mediated by a particular protein or small group of proteins. Benga and co-workers did see bands in a gel that corresponded to water transporters, and were the first to do so. In 1988 Peter Agre and co-workers in Baltimore, USA, while working on the rhesus blood group antigens, purified a "new" membrane protein that they called CHIP 28 (channel integral membrane protein of molecular weight 28k). At the time they had no idea of its function. In 1992 came the definitive experiment, that was done, according to Peter Agre in his Nobel Lecture, after much discussion with colleagues about the likely candidate function of their 'orphan' protein. In a paper published in 1992 in *Science* Agre and his group describe how CHIP28 has the properties of a water channel protein. In 1993 the name of the protein was changed from CHIP28 to aquaporin 1. It became obvious that one of the labelled bands observed by Benga's group (the one in the region of molecular weight ~35,000 to ~60,000) corresponds to glycosylated CHIP28 (aquaporin 1). So Benga and co-workers did first see bands in a gel that corresponded to water transporters, and were the first to do so. The "mercury labelling" experiments were confirmed and extended in Cluj-Napoca by Benga's group and the results were published in 1986 in the *European Journal of Cell Biology*, another international journal. The work was reviewed by Benga in subsequent years in international series and even as a chapter in a book on water transport edited for a well-known US-based publisher. Agre's group did include a reference to Benga's work in their *Science* paper; but this reference was only to a 1983 paper on protease resistance of "water channels" (which was relevant) and not the pertinent 1986 *Biochemistry* paper, or even the subsequent publications. The report of the recent exciting finding of possible involvement of aquaporins in epilepsy, published in 2005 in *Proc Natl Acad Sci USA* by a group including Agre failed to cite Benga and Morariu's novel and startling report in *Nature* in 1977.

Key words: water transport, water channel, water channel protein, red blood cell, nuclear magnetic resonance, epilepsy, Nobel Prize.

INTRODUCTION: A DIVIDED SCIENTIFIC WORLD

The story of the discovery and understanding of the aquaporins is an intriguing one and the work is thoroughly deserving of the 2003 Nobel Prize in Chemistry, which was awarded to Professor Peter Agre.

However, there have been some curious oversights in this tale.

Professor Gheorghe Benga has conceived of many clever experiments both before his 1986 *Biochemistry* paper (11) and since then; today his dedication to science is unabated. He was a stellar student of Medicine at the "Iuliu Hatieganu" University of Cluj-Napoca in Romania (where he also obtained a Ph.D. in Medical Biochemistry), and (after

Abbreviations: NMR: nuclear magnetic resonance;
pCMBS: p-chloromercuribenzenesulfonate;

graduating in Chemistry at the "Babes-Bolyai" University of Cluj-Napoca and post-doctoral work with Professor Dennis Chapman at the University of London) was appointed at a young age to head a new Department of Cell Biology at his Alma Mater. So, at home in communist Romania in the 1970's - 1980's, behind the "iron curtain", people were perhaps not surprised that he was doing world-class science. But, herein lies the problem: did the rest of the scientific world know? Or, as I am about to explain, did the rest of the world *want* to know?

The 1986 *Biochemistry* paper (11), to which I refer, describes results of experiments that, as far as most scientists are now aware, provided the first visual and tangible evidence that the very

rapid water exchange that occurs through the membranes of human red blood cell is mediated by a particular protein or small group of proteins.

‘THAT’ EXPERIMENT

It is now common knowledge that the channels that permit the selective flow of water across cell membranes (aquaporins) are ubiquitous in Biology; but they were only definitively identified a mere 13 years ago. Why did it take so long for them to be discovered? In a sense it was because they are ubiquitous; i.e., often the easiest way to identify something is to compare two similar objects (in this case cells) and then determine what is it that makes them different. This of course was not possible for water channels as almost all cells have them. In 1988 Peter Agre and co-workers, while working on the rhesus blood group antigens, discovered a “new” membrane protein that they called CHIP 28 (channel integral membrane protein of molecular weight 28k). At the time they had no idea of its function (12). In 1992 came the definitive experiment (15). It was done, according to Peter Agre in his Nobel Lecture (1), after much discussion with colleagues about the likely candidate function of their “orphan” protein, noting in particular that both the kidney and the red blood cell are well known to be very permeable to water.

Even during the communist period Gheorghe Benga travelled extensively overseas to conferences, and carried out experiments in several foreign laboratories. He was very productive during these excursions so he had little difficulty in gaining agreement from various hosts for his short-term research stints; on the other hand foreign scientists also made research visits to his department. (Later I witnessed his extraordinary energy when it came to be my turn for collaboration; in this work we made heavy use of NMR spectroscopy for biophysical and biochemical studies of red blood cells.) It was when Ross Holmes from the University of Illinois, Urbana, went to Cluj-Napoca in 1985, that Benga’s group did the “mercury labelling” experiments, prompted by some insights that were based on decade-long work on red blood cell water permeability, and Benga’s encyclopaedic knowledge of the literature on water transport. After the work was finished it was published in *Biochemistry*, a US-based journal (11).

The background to this experiment is as follows: it was known from Macey and Farmer (14) that *p*-chloromercuribenzenesulfonate (*p*CMBS) inhibits water transport across the membranes of human red blood cells; it supposedly did this by binding to “deeply buried” sulfhydryl groups on membrane proteins. So it was a matter of buying the commercially available radio-active-mercury [²⁰³Hg] labelled *p*CMBS, using it in an experiment of specific inhibition of water transport and identifying the membrane proteins binding the inhibitor. This was done on red blood cells and resealed ghosts, the clearest result being obtained with resealed ghosts (see Fig. 2a in ref. 11) that were first preincubated with a reagent (N-ethyl maleimide) that does not inhibit the water transport but reacts with “surface-accessible” sulfhydryl groups; then the incubation with the labelled *p*CMBS was performed under conditions of specific inhibition of water transport (measured by an NMR technique). After this the red blood cell membrane preparation was dissolved in the detergent sodium dodecyl sulfate (SDS), the sample applied to an electrophoresis gel and a potential difference applied across it. The various proteins were separated in the gel according to their ionic mobility and hence molecular weight. Then to detect where the radioactivity was located, the gel was sliced into bands and the radioactivity of each was “counted”. There was indeed labelling of proteins in various regions of the gel and these regions correlated with bands of Coomassie blue dye that stains proteins. The authors deduced that, because *p*CMBS inhibits water transport, and it is bound to proteins in the red blood cell membrane, these proteins are the much sought after water transporters. A closer look at the results (Fig. 2a in ref. 11) revealed that some proteins of molecular weight ~35,000 to ~100,000 were labelled, with two peaks: one corresponding to the “band 3 protein” with a molecular weight of ~80,000 to ~100,000 and the second broader peak covering the molecular weight region ~35,000 to ~60,000. Polypeptides migrating in these regions had already been identified in other transport functions, notably anion exchange (“band 3 protein” with a molecular weight of ~80,000 to ~100,000) and the transport of glucose and nucleosides (“band 4.5 proteins” with a molecular weight ~55,000). However, there was no significant inhibition of water transport by inhibitors of anion, or glucose and nucleoside transport. Therefore, the authors concluded, “a

minor protein that binds PCMBs is involved in water transport" (11).

It has subsequently been shown that one of these labelled peaks (the one in the region of molecular weight ~35,000 to ~60,000) corresponds to glycosylated CHIP28 (aquaporin). So Benga et al. (11) did see bands in a gel that corresponded to water transporters, and were the first to do so. The "mercury labelling" experiments were confirmed and extended in Cluj-Napoca, this time in collaboration with John Wrigglesworth and Anthony Brain of King's College, University of London; and the results were published in another international journal, the *European Journal of Cell Biology* (10). The work was reviewed by Gheorghe Benga in subsequent years in international series (3, 5-7) and even as a chapter in a book on water transport edited for a well-known US-based publisher (4).

SERENDIPITY

The curious thing to note about this scenario is that Gheorghe Benga was "out looking for the water transporter" (10), while Peter Agre was not (12). Because Peter Agre and his group had isolated a protein in abundance they surmised that it would be important; and because it was easy to purify, in their hands, they could start applying modern laboratory methods to determine its function. The requisite methods in protein chemistry and molecular biology, of course, were readily available in the US at the time; nevertheless it still took the team 4 years to do the definitive test, the loss of osmotic resistance in frog eggs expressing the protein. Then the name of the protein was changed from CHIP28 to aquaporin (1).

SCHOLARSHIP

When most scientists make a discovery, of something even much less profound than aquaporins, they scour the literature for insights and possible evidence of similar findings. This information is used to set the scene in a description of the work in the Introduction of the paper in which the results are first presented, and in subsequent papers on the topic. So, in 1992 there is evidence that Peter Agre did the same. Of course he need not have known about *all* of Gheorghe Benga's work on water transport; after all he was not working in the field of water transport! But he and his group did include a reference to Benga's work in their

groundbreaking paper (15); but this reference was only to a 1983 paper (9) on protease resistance of "water channels" (which was relevant) and not the pertinent 1986 *Biochemistry* (11) paper, or even the subsequent publications (3-5).

LITERATURE SEARCHING

It is easy, today, to conduct a Web of Science (or similar) literature search using one's own computer. In my case it takes place via the University library that has the appropriate subscription to this service. Searching is done in a way that was not routine in 1992. Now one simply logs into the search site and types in a keyword, and then presses the "search button". I did this on 29/10/05 for the topic "Water Transport", and scored 3592 "hits"! (Note, I fully expect that any reader would be able to verify all the "search" findings I present here.) I then typed in "BENGA G*" and noted, amongst the 90 references (not all are by the G Benga of this discussion), that the *Biochemistry* paper of 1986 (ref. 11) has been cited 56 times. At a single stroke of the computer keyboard it is possible to see who amongst these 56 authors has cited the paper. Peter Agre has *never* cited the 1986 Benga paper (11); at least not amongst any of the journals abstracted by the Institute for Scientific Information (ISI; Current Contents). So the paper had no *formal* (in terms of warranting citation) impact on any of the work in the Agre laboratory.

I then decided to determine if Peter Agre had recently (since 1992) cited Professor Benga's papers and on working back from 2005 I found no paper until a 1994 review in which only a single 1984 review paper of Gheorghe Benga is cited. Of course, scientifically, there was no longer any need for Peter Agre's group to do this as the science of aquaporins was progressing in leaps and bounds via modern methods of molecular biology and no *p*CMBS or related experiments were used.

It is another matter, when *reviewing* the topic of the discovery of the aquaporins to review the whole literature as far back as is deemed "scholarly"; paying attention to work on, say, *p*CMBS. Equally it might have been expected to include the 1986 *Biochemistry* paper (11) since in a good sense this is the culmination of many preceding experiments with this reagent. While this paper may have been overlooked in the 1994 review, modern literature searching carried out to

take stock of the field in 2001 should have turned it up, e.g., in ref 2. Certainly, a 2005 Web of

Science search on “WATER AND RED AND CELL” and the author names, BENGA G* or AGRE P* turns up 28 and 30 references, respectively.

DÉJÀ VU AND THE FUTURE

Perhaps Gheorghe Benga could be permitted just a tinge of déjà vu when the report of the recent exciting finding of possible involvement of aquaporins in epilepsy (13) failed to cite his and Dr Morariu's novel and startling report in *Nature* in 1977 (ref.8). This work, that in a sense was well before its time because no mechanistic explanation was then available, was on the prolongation of water exchange times in the red blood cells of epileptic patients.

I can recommend to you, for a scholarly analysis of the discovery of aquaporins, by someone involved in the field for their whole career, the recent review by Professor Gheorghe Benga (7). In addition we celebrate science and the advances that are made in all places of the world where people turn their minds to difficult questions about Nature. If my present Letter achieves nothing else I hope that this earlier work (7, 10, 11) attracts its proper, justified, acknowledgement in the field of aquaporins, including their involvement in epilepsy. But I actually hope for more: that the scientific contributions of Gheorghe Benga should be acknowledged internationally not simply within Romania. Given the ground swell of support that this idea is receiving at some World conferences (17, 18), I predict that my voice will simply be one of thousands (16).

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