



In memoriam

In memoriam Peter Mazur – Cryobiologist

Cryobiology has lost one of its great pioneers. Born on March 3rd, 1928, Peter Mazur died peacefully at home in hospice care in Oak Ridge, Tennessee, USA on December 30th, 2015. He fought a two year battle with lung cancer and had the upper hand until December. He was doing his beloved cryobiology until his last week.

Peter graduated magna cum laude from Harvard University in 1949, and obtained his PhD in 1953. After serving with the United States Air Force for four years he spent 2 years as a Post-Doctoral Fellow at Princeton University. In 1959 he moved to Oak Ridge, Tennessee to join the Biology Division at the Oak Ridge National Laboratory (ORNL), where he began a distinguished career spanning nearly six decades and producing more than one hundred and seventy scientific papers – work that helped cryobiology to achieve the status that it enjoys today.

In 1969 Peter chaired a CIBA Symposium, “The Frozen Cell”, in London. It was attended by an impressive international panel of scientists. Referring to the work of Audrey Smith and James Lovelock at the National Institute for Medical Research, UK, Peter's opening remarks included the following:-

“So persuasive were the findings of Smith, Lovelock and their colleagues that other investigators have tended to assume that the freezing procedures developed for sperm are also optimal for cells in general and they have tended to assume that Lovelock's explanation of freezing injury and protection holds for cells in general. The central question I would like to pose for our consideration is: to what extent are these assumptions valid?”

The basic assumption in question was that freezing injures cells because it subjects them to concentrations of electrolyte above a critical mole fraction and because thawing subjects them to dilution. Peter emphasized that Lovelock's analysis ascribed most freezing injury to a single cause and was not a result of ice formation *per se*. He continued, “some of us believe that there are *at least* two factors responsible for injury, and that one of these is in fact ice formation within the cell”.

Peter's group soon showed that plots of cell survival versus cooling rate typically exhibit a peak at a cooling rate that is a characteristic of each particular cell and cryoprotectant combination. Peter wrote, “The existence of an optimum cooling velocity must mean that survival is affected by at least two factors that depend oppositely on cooling rate”. Thus was the “two factor hypothesis”

born, supported by a mass of experimental evidence for a range of cell-types and cryoprotectants. In fact Peter envisioned additional mechanisms one of which was the reduction of temperature *per se* – or chilling. It was also shown that survival was influenced by the warming rate, rapid cooling favoring rapid warming.

It is difficult to overstate the importance of this analysis. Prior to its formulation, the development of cryopreservation techniques was an entirely empirical exercise but Peter's fundamental insight provided a firm basis for much subsequent research, including the optimization of specific cryopreservation processes. An early development of practical importance was the design of effective cryopreservation processes for haemopoietic stem cells, crucial for the clinical use of high dose X-ray- or chemo-therapy. Peter demonstrated the relationship between the cryoprotectant (glycerol in his experiments), its concentration and the optimal cooling/warming rate for the cryopreservation of mouse haemopoietic stem cells. The cryoprotectant dimethyl sulphoxide (DMSO) is more commonly used for such stem cells nowadays but in other respects the process is just as Peter's group developed it. The importance of this work to the science of cryobiology is simply tremendous: it is difficult indeed to know where the subject would be now – indeed whether it would still exist – without Peter's contribution.

Peter's methodical approach proved invaluable when a new challenge arose in 1971: another laboratory reported that mouse embryos could be cryopreserved using polyvinylpyrrolidone (PVP) as the cryoprotectant and cooling very rapidly. Peter's group could not repeat this result. Peter's response was characteristic – he invited the author of the report to join the group at ORNL where they would approach the problem in the light of the cryobiological fundamentals that Peter had just established: to examine the effects of cryoprotectant, cooling rate and warming rate. They found that, in the presence of DMSO or glycerol the optimum cooling rate was very low so that slow warming would be expected to give the best survival. Experiment showed this to be so and led to the successful cryopreservation of mouse embryos – a discovery of great practical importance for many branches of biology and subsequently to clinical medicine.

Encouraged by this success Peter accepted the challenge to develop a procedure for the cryopreservation of *Drosophila* embryos in order to facilitate the maintenance of the thousands of mutant lines used in genetic research. He and Peter Steponkus independently attacked this problem. These embryos are encased in a waxy vitelline membrane that renders them waterproof (and

cryoprotectant-proof). A method was developed to permeabilize the vitelline membrane by exposure to a carefully formulated mixture of organic solvents that the embryos would tolerate. But that was not sufficient to solve the problem: the permeabilized embryos were found to be extremely sensitive to chilling even in the absence of ice: chilling injury was the third potential factor in freezing injury that Peter had noted way back in 1969. They discovered that the chilling injury was reduced by very rapid cooling but that increased the risk of intracellular freezing! To overcome this, the embryos were exposed, in two steps, to very high concentrations of a permeating cryoprotectant that increased the internal concentration, largely by dehydration. The embryos were then plunged into nitrogen slush at -205 deg C to achieve cooling and warming rates of $\sim 100,000$ deg C/min. producing a glass rather than ice. The result was 12% embryo survival to hatching and 5% of larvae developing into adult flies. The degree of overall success was limited but the sequence of problems and their step-by-step solution provided a fascinating lesson for cryobiologists and provided an important insight into chilling as a third factor in freezing injury. These experiments were not without other difficulties. After an initial success and opening of the champagne bottles, it was a year before they could be replicated; the initial success being the result of a timing error in the embryo development.

In 1981 Peter returned to the basic question he had posed in 1969 – Is the freezing injury to slowly cooled erythrocytes caused by the rise in solute concentration or the reduction of the available space. Peter pointed out that “no experiment in which cells were suspended in isotonic saline and then frozen, whether or not penetrating cryoprotectants were also present, could possibly distinguish between an effect of rising concentration and an effect of reduced volume of liquid since these changes were linked, one-to-one by the phase diagram of the system in question”. To separate the effects of these two possibilities Peter designed an ingenious experiment that involved freezing and thawing erythrocytes suspended in solutions having the same ratio of salt to glycerol but differing in total solute concentration to produce a range of tonicities from $0.6 \times$ isotonic to $4 \times$ isotonic. He wrote:-

“The results proved unexpected and even astonishing for they show that the survival of the slowly frozen erythrocytes is far more dependent on the fraction of water that remains unfrozen than it is on the concentration of salt (NaCl) in that unfrozen water.”

This was the “unfrozen fraction” hypothesis of freezing injury. An active debate ensued. The experiments were ingenious but unavoidably there were confounding factors – notably the fact that each group of cells started the experiment at a different volume. The four papers describing this work are classic Mazur papers: the writing is immaculate; every detail is included; each assertion is exhaustively justified; and the discussion is penetrating.

In the late 80's Peter began a 10 year collaboration with John Critzer's lab at Methodist Hospital, Indianapolis, IN. This initiated a long period of work on sperm in addition to embryos. Many grants and papers from this period begin with the word 'Fundamental - - -'. Peter was less interested in successfully freezing any given cell line and more interested in the fundamentals which could be applied to all cell lines. On the other hand, funding agencies were often interested in success with a specific cell line. Following his success with *Drosophila*, the malaria community became interested in the freezing of mosquito embryos to maintain their mutant lines and Peter was funded to tackle this problem. Alas, neither the *Drosophila* approach nor many others proved successful.

In 1998 ORNL wanted to close the building containing Peter's lab and everyone was retired and unceremoniously kicked out. However, being only 70, Peter was not interested in retiring. The University of Tennessee, Knoxville, was delighted to accept Peter along with his lab equipment and grant money and thus began a new phase of his career. Peter often lamented the retrenchment of the ORNL Biology Division from its glory years.

Around 2000 Peter returned to intracellular ice formation, ice recrystallization, and the importance of warming rate on survival. His thesis was that the warming rate was as, or more, important than cooling rate. This work culminated in his ground breaking work of the past few years using lasers to achieve ultra-rapid warming rates. Applied to mouse oocytes and embryos, he was able to achieve warming rates 10–100 times faster than by conventional means. This allows the use of less concentrated and less toxic cryoprotectants while maintaining high survival. His last (unfinished) grant application was directed to using the laser ultra-rapid warming method to cryopreserve some difficult cell lines which have not previously been successfully and reproducibly cryopreserved, e.g. mouse sperm.

Many scientists have had the good fortune to train and work in Peter's laboratory over the years. We mention just a few names: Stanley Leibo, Bill Rall, Ray Rajotte, John Armitage, Uli Schneider, Igor Katkov, Chihiro Koshimoto, Keisuke Edashige, Shinsuke Seki, Bo Jin, Fritz Kleinhans, and most recently, his current post-doc, Estefania Paredes.

I (DEP) met Peter in 1962 when I made my first trip to cryo-labs in the USA. Peter could not have been more welcoming and it was not difficult to recognize his lab as the true center of cryobiological research in the world. I particularly remember him proudly showing me his transfer standard thermometer, indicating that everything in this lab was securely grounded in physical science. The Society for Cryobiology was formed in 1964 and thereafter I, and many others, enjoyed annual discussions of cryobiology, also involving Stanley Leibo of course, and often over dinner at meetings and illustrated by impromptu sketches drawn on paper table napkins. Much later we had detailed discussions of Peter's “unfrozen fraction” hypothesis, about which we had differing views but we were able to debate openly without the slightest effect on our personal relationship – something that was entirely consistent with Peter's personality. Somewhat later, when I was having funding difficulties with the Medical Research Council in the UK, I was delighted when Peter was selected to be a member of our site visit committee and I am sure that our survival owed a lot to Peter's input on that occasion.

I (FWK) happen to have been present during the ‘Russian Years’. Among Peter's many talents were giving driving lesson to Igor Katkov for his American driver's license. Peter survived and Igor got his license! This was typical of Peter's generosity. More recently, there was Peter's Russian lab assistant, Irina Pinn. She had a special talent for pulling Peter's ‘republican/conservative’ string most every day, engendering many lively discussions. Peter's (step) son-in-law, Richard Dawson, was equally adept at tugging on his conservative ‘strings’.

Peter's lab was not a place of all work and no play! Indeed, cryobiology was not ‘work’ for Peter. It was his passion. Rumor has it that he was still clutching his latest NIH grant application in his left hand and new experiments for his post-doc in his right when he died. At national meetings his presence was always felt. Seemingly no matter the topic, he had penetrating questions after every talk. He was a walking encyclopedia of cryobiology with a steel trap mind. On many an occasion I (FWK) would ask him a question about a paper that was, say, 10 years old. Yes he would say, the authors were right about points *a* and *b*, but wrong about *c*. Later, I might look at the paper in Peter's original copy of the Journal and

there would be his comments in the margin, perfectly remembered by him 10 years later. Indeed on those rare occasions when I found an interesting paper that he had not read his first question was always “Where did you find that”!

Over his long and brilliant career, Peter Mazur received numerous honors and awards: he was one of the first three recipients of the Society for Cryobiology's Fellowship Medal (2005); he was a long-serving member of the Board of Governors of the Society for Cryobiology, its President from 1973 to 1974, and a Member of the Editorial Board of the Journal CRYOBIOLOGY from 1967 to the present. At ORNL he became a Corporate Fellow in 1985 and was chair of the ORNL Corporate Fellows Council from 1994 to 1996. In 1993 he received the Distinguished Service Award from the AATB. Of his 170+ scientific publications 4 were named as Citation Classics by the Institute for Scientific Information. He enjoyed his work as a member of the Space Science Board of the National Academy of Sciences (1975–1977) where he was chairman of the Exobiology Committee and dealt with the question of life on Mars and the Viking Missions.

He will be dearly missed by all of us who had the good fortune to be his students, post-docs, and colleagues and to learn at his hand and share his friendship. Peter Mazur is survived by his son, Timothy; his daughter-in-law Kathy; his step-daughter Jennifer Frame Dawson, his step son-in-law Hal Richard Dawson, and his

five grandchildren. Drusilla Stevens Mazur, his first wife, died in 1982. His second wife, Sara Jo Boling Frame Mazur, died in 2003.



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