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TURNING POINT ARTICLE

PLANT PROTOPLASTS

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What led me in 1960 to begin work to develop an enzymatic procedure for the isolation of plant protoplasts? The truth is that I initially had no idea that I wanted to isolate plant protoplasts! I arrived in 1959 at the University of Nottingham as a lecturer in plant physiology. I had already completed 3 yr as a Civil Service Commission postdoctoral fellow in bacterial chemistry, after receiving my Ph.D. in plant biochemistry from the University of Bristol in 1956 for studies on the synthesis of amino acids and proteins in barley. Two viewpoints had impacted on me from these 6 yr of research. The first was that the whole plant was too complex for detailed biochemical analysis and that experimentally it would be better to reduce it to the cell level and then reassemble it to relate biochemical studies to the whole plant. The second was an appreciation from studies with bacteria that single plant cells and small groups of cells would be much more amenable to biochemical analysis and cell biological studies. During this time, I had also been interested in the development of new analytical procedures and had experienced the way in which their introduction had opened up new research areas in the biological sciences. From the results of the first year of my Ph.D. I had published with my supervisor an improved procedure for the determination of amino acids with ninhydrin (Yemm and Cocking, 1955), which was to play a key role in the quantitative analysis of the amino acid composition of proteins. Also, by researching with bacteria, which produce results more quickly than plants, I had already published two significant papers and was keen to produce isolated cells from plants with the aspiration of developing a plant cell 'bacterial' type of culture system in which plant cells would divide, separate and produce a culture of single cells. The approach I adopted was to investigate the use of chelating agents to isolate cells from the rapidly elongating region of the roots of tomato seedlings. This established that cell separation was largely dependent on Ca^{2+} in the middle lamella. However, although extensive cell separation was achieved, no division of the isolated tomato root cells was observed and it became evident that the chelating agents were negatively impacting on cell physiology and biochemistry. Chelating agents were therefore of little use; my thoughts turned to the possibility of breaking down not just the middle lamella but the cell wall itself in order to release the protoplast from within the cell wall. I had read about the isolation of bacterial and fungal protoplasts by the use of enzymes degrading their cell walls and, probably more importantly, I had had discussions with workers on bacterial protoplasts at the Microbiological Research Establishment, Porton when I had been a postdoctoral fellow. My training in plant biochemistry told me that I would have to use cellulases degrading cellulose rather than lysozyme. Also, I knew that it *was* possible to isolate plant protoplasts by physically breaking the cell wall, provided the protoplast had been plasmolyzed away from the cell wall. In my undergraduate practicals at the University of Bristol I had cut through pieces of plasmolized beetroot and observed the release of protoplasts from the ends of cut-through cells.

As I have recounted, and illustrated with a picture of the first protoplasts isolated in May 1960 from tomato seedling root tips using *Myrothecium verrucaria* cellulase (Cocking, 1983), this led me to survey a wide range of commercially available cellulase preparations for their ability to isolate plant protoplasts. Seedling roots were chosen because material could thereby be readily obtained with cells at different known stages of differentiation, with minimal problems of penetration of the enzyme. Many commercially available enzyme preparations were tested, all without success. While this work was in progress, I read of the studies of D. R. Whitaker (National Research Laboratories, Ottawa) on the purification of cellulase from *M. verrucaria*. He generously provided me with a few grams of his enzyme preparation and, in his covering letter, dated 25 November 1959, said 'In as much as the cellulose in cell walls tends to be highly crystalline, I should think its degradation would be a slow process—quite apart from accessibility factors due to other components of the wall acting as a physical barrier.' Thinking that my idea of using cellulase might therefore be ruled out by such physiochemical factors, I put his sample at the bottom of the deep freeze. Only when everything else had failed did I test his preparation; it released protoplasts! (Cocking, 1960).

Microscopically observing protoplasts being released from the rapidly elongating region of the roots and from the highly meristematic region was exciting, and it was possible to examine the inside of the protoplast with extra clarity because there was no cell wall. The more I looked at protoplasts the more I appreciated the indispensability and fundamental importance of the plasma membrane of the protoplast. In this respect it is interesting to recall that whether cells were inevitably bounded by a membrane was a question that arose because the solid walls that characterized plant cells could not with certainty be identified in animal cells. Also, as recalled by Henry Harris in his recent perceptive analysis and meticulous historiography of the cell doctrine (Harris, 1999), the distinction between the plant cell wall and the cytoplasmic membrane was only finally established by the classical plasmolysis

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studies of Ernest Overton towards the end of the last century. When beginning to work with isolated protoplasts in the early 1960s I read carefully the earlier studies on protoplasts and realized that the groundwork for much that I was thinking of now doing with enzymatically isolated protoplasts had already been laid towards the end of the nineteenth and early in the twentieth century (Cocking, 1965). In 1880 Von Hannstein had proposed that the basic unit be called a protoplast; in 1892 Klercker described 'Eine methode zur isolearung lebender protoplasten'; and this mechanical method of isolating protoplasts was refined by Plowe in 1931, who isolated protoplasts from the cells of onion epidermis by plasmolysis in sucrose. In her studies, highly vacuolated protoplasts were reduced to about half their original volume and a blade passed between the end walls and protoplasts of cells, enabling intact protoplasts to be released. These mechanical methods for the isolation of plant protoplasts, although providing suitable material for the investigation of the osmotic behavior of isolated protoplasts, were always limited by the small number of protoplasts that could be isolated and by the fact that protoplasts could only be isolated from elongated, highly vacuolated cells. It is interesting to recall that Kuster (1935) in his book *The Plant Cell* used the term 'gymnoplasts' to describe naked protoplasts. Indeed there was a tendency in the late 1950s to describe protoplasts as naked cells and my original title for the proposed enzymatic isolation procedure paper was 'A method for the isolation of naked cells and vacuoles'. Such a title, containing the word 'naked' as it did, was not acceptable to my then Head of Department, who read through all papers before they were submitted for publication, and the title was changed from 'naked cells' to 'protoplasts'! It is also interesting to recall that even as late as 1967 the distinguished Swiss botanist A. Frey-Wyseling was suggesting that protoplasts should be called 'gymnoplasts' based on Kuster (1935). Fortunately, I was able to override this claim on historical precedence (Pojnar and Cocking, 1968); if his viewpoint had been upheld we would have had bacterial and fungal protoplasts and plant gymnoplasts.

The problem with all the protoplasts that had previously been isolated from highly vacuolated mature plant cells using mechanical methods of isolation was that they did not undergo further growth and development. They were excellent for studying osmotic relationships and occasionally they would fuse with one another but only under erratic and unreproducible conditions. My interest therefore centred on whether enzymatically isolated protoplasts would re-synthesize a wall and undergo further development; if they did not they would remain cytological curiosities suitable only for osmotic relationship studies and various micromanipulations, which would not advance plant developmental biology and my vision of the re-synthesis of plants from single cells. This investigation raised several basic cytological questions. Would mitosis and cytokinesis occur in the absence of the wall or would protoplasts first re-synthesize a wall and then divide? The first clues came from studies on the properties of protoplasts isolated from root meristems. There was evidence that protoplasts first developed some form of primary cellulose wall and then acquired some aspects of the behavior of cells in suspension culture (Cocking, 1969). But it was not until nearly 10 years later that the division of cells originating as enzymatically isolated tobacco mesophyll protoplasts was reported (Takebe et al., 1968). Looking back at this gap of nearly 10 years it is useful to identify the reasons for it and whether this delay was as much a negative as it might at first appear to be. In some ways my

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isolation of protoplasts using *M. verrucaria* cellulase in 1960 was ahead of the then technology of plant cell-wall-degrading enzyme production. Insufficient enzyme was available to me to attempt anything more than the isolation of protoplasts from roots and their culture on a miniscale in special microculture chambers, including studies on their responses to growth substances and progressive vacuolation (Cocking, 1961). Indeed it was not until 1968 that the use of commercially available *Trichoderma viride* cellulase, produced in Japan for baby food and biscuit manufacturing, enabled Japanese researchers to undertake the large-scale isolation of protoplasts from tobacco leaves (Takebe et al., 1968). This led to the successful culture, following cell wall re-synthesis, of isolated tobacco leaf protoplasts (Nagata and Takebe, 1970) and, following this, to the regeneration of whole plants (Takebe et al., 1971).

At Nottingham from 1960 onwards we had the basic technology, but not the scale-up capability, and we were held back from doing extensive large-scale experiments with leaf protoplasts. Fortunately we discovered, by accident in 1963, that by using the readily available commercial pectinase we could isolate protoplasts in very large numbers from the parenchymatous locule tissue of immature tomato fruit in a plasmolyzing medium. We were in fact trying to obtain separated cells but released protoplasts instead because the cell walls of the locule tissue were mainly pectin (Gregory and Cocking, 1965). Our apparatus for the mass production of tomato fruit protoplasts provided us with large quantities of these isolated protoplasts throughout the 1960s. This enabled us to undertake studies in which the accessibility of the plasma membrane meant that experiments could be designed to investigate and manipulate the properties of this membrane in a way that was not possible with walled cells. This then led us to an interest in the fusion of the plasma membranes of protoplasts, but using root protoplasts because fruit protoplasts were too highly vacuolated. With hindsight, I do not think that I would have pioneered, with the

help of the excellent students and postdoctorals who came to work with me, the interaction of viruses with protoplasts, cell wall synthesis by protoplasts or ultimately the fusion of protoplasts for somatic hybrid and cybrid production if it had been possible to isolate protoplasts using commercially available cellulases in the early 1960s. Also, at that time I was personally more interested in light microscopy and electron microscopy, having worked with Irene Manton at the University of Leeds, in Göttingen with Heinrich Matthaei using his institute's electron microscope and then with my own electron microscope at Nottingham for investigating the cell biology of protoplast systems, particularly in relation to the behavior of the plasma membrane. This led to investigations with a Polish visiting researcher, Edward Pojnar, which showed that protoplasts isolated from tomato fruit locule tissue readily regenerate a new cell wall (Pojnar et al., 1967). Cell wall regeneration is probably one of the most significant things that protoplasts do. What I regard as one of my most interesting papers, which I have never seen cited, described that in tomato locule tissue the cells secreted cell-wall-degrading enzymes releasing protoplasts, protoplasmic units and vacuoles; no addition of cell-wall-degrading enzymes was required; the protoplasts were a gift of Nature! This study (Cocking and Gregory, 1963) showed that these protoplasmic units were compartments of the protoplast that had become separated. The protoplast was like a jigsaw puzzle in which the separate pieces could have an independent existence; the best way for a cell to die gradually?

My interest in the behavior of the plasma membrane at the surface of tomato fruit protoplasts, which were available reproducibly in large quantities, included studies of cell wall synthesis, with detailed investigations using freeze-etching procedures of the uptake of ferritin and latex particles and the role of endocytosis (Cocking, 1970). These studies led our protoplast research in two major directions (Cocking, 1972). One was from the observation that cell aggregates were formed if tomato fruit protoplasts were kept in contact with each other during cell wall regeneration. The variation in cell shape that was evident in these cell aggregates was interesting and it was suggested that by studying the variation in shape under different conditions it might be possible to obtain a fuller understanding of the factors controlling cell shape in plant tissues. It was also suggested that the ability to form cell aggregates from regenerating protoplasts might be significant in relation to investigations of the factors influencing the development of plant chimaeras (Pojnar and Cocking, 1968). Little has happened in this respect in the intervening years, probably largely because the development of methods for the transformation of protoplasts, plant regeneration from a wide range of protoplasts and the production of transgenic plants, together with the advent of protoplast fusion and somatic hybridization, ushered in the era of plant genetic manipulations. Basic cell biological studies with protoplasts have been somewhat neglected. However, studies on the uptake of macromolecules and particles ushered in studies on the use of isolated protoplasts in plant virology. It was perceived that the use of protoplasts in these studies had two major advantages over the use of plants themselves. First, the protoplasts can be synchronously infected with the virus, and second, because of the high proportion of infected cells, it is possible to carry out meaningful biochemical and molecular investigations, particularly of the early stages of infection. The first steps arose from an electron microscopic study of the initial stages of infection of isolated tomato fruit protoplasts by tobacco mosaic virus (TMV) which provided evidence for a pinocytotic uptake of TMV, but the subsequent fate of the virus particles remained in doubt (Cocking, 1969). Following these observations, a quantitative sectioning procedure providing electron microscopic evidence for infection was undertaken; after the disappearance of virus from pinocytotic vesicles and the regeneration of these isolated protoplasts into cells, virus appeared in the cytoplasm in aggregates characteristic of TMV infection in these cells (Cocking and Pojnar, 1969). As a consequence, one of the principal difficulties of the lack of a model system, in which cells under study can be simultaneously exposed to virus particles and become infected, had been overcome. Facilitated by the commercial availability of *Trichoderma veridi* cellulase, enabling the large-scale isolation of leaf protoplasts, these studies were extended to the infection of tobacco mesophyll protoplasts by TMV (Takebe and Otsuki, 1969). This led to a series of ongoing studies by numerous other researchers using protoplast systems in plant virology (Murakishi et al., 1984). These were also a prelude to studies on the uptake of foreign genetic material by plant protoplasts, including extensive studies on the uptake of DNA, viruses, organelles and microorganisms, even extending to the uptake of rhizobia into leaf mesophyll protoplasts and of blue-green algae cells into culture cell protoplasts (Cocking, 1977). These studies heralded the development of a system using protoplasts for cell transformation paralleling bacterial transformation, culminating in the demonstration that the *Agrobacterium* tumor-inducing (Ti) plasmid was able, in the absence of any agrobacteria, to transform *Petunia* protoplasts, providing the first proof of the independent role of the Ti plasmid in this respect (Davey et al., 1980). This paved the way for the production of transgenic plants by DNA transformation of protoplasts, including the production of fertile transgenic rice plants following electroporation of rice protoplasts with chimeric plasmids (Zhang et al., 1988).

Just as my beginning research in 1959 at the University of Nottingham had been a 'turning point', so 10 years later in 1969 there was another turning point. Three significant events in that year were to set the pathway for the general direction of my research on plant protoplasts for the next 20 years. I was invited to accept the Chair of Botany and Headship of the Department of Botany at the University of Nottingham, where I had already been researching and teaching for 10 years. This ensured a period of stability and ongoing association with high quality research colleagues and an ability to undertake research that I wanted to do and not what some research committee thought I should do. Also that same year I was invited to participate in a Rockefeller Foundation Discussion Meeting at the Villa Serbelloni in Bellagio, Italy, on the potential application of plant cell and tissue culture to crop plants. The Nobel Laureate Frank Macfarlane Burnet joined us briefly for one of our discussion sessions. Later in his book *Genes, Dreams and Realities* he wrote: 'What the botanists had in mind was the possibility of an incomparably wide range of hybridizations if a means could be found to fuse somatic cells of almost unrelated species and persuade such unnatural hybrids to develop into complete plants. If cells of man and fowl or man and mouse can produce composite cells which can multiply in tissue culture, why should not a cell of high-bearing rice hybridize with a desirable strain of sweet potato? And if a tobacco or begonia single cell can produce a complete plant of its proper type, why should not the hybrid cell produce a plant with the virtues of both its parents? So far as I am aware, no such artificial fusion of plant cells has yet produced a complete

composite plant, but I know that a variety of possible approaches is being explored.' (Burnet, 1971). This Discussion Meeting was particularly timely because, whilst regeneration of whole plants from cultured isolated protoplasts had not yet been achieved, fresh opportunities were arising, particularly in relation to gene transfer between species using novel procedures, especially the use of isolated plant protoplasts (Nickell and Torrey, 1969). Meanwhile, in the UK, the Agricultural Research Council (ARC) also recognized that fresh opportunities were arising for gene transfer using protoplasts between species using novel procedures, and an ARC Research Group under my leadership was established in the Department of Botany. This ensured 16 years of ongoing funding which led to the development of plant genetic manipulations in the age of plant biotechnology. In 1969 our first objective was to obtain induced reproducible fusion of cereal protoplasts and this was achieved 1 yr later using sodium nitrate as the fusogen (Power et al., 1970), followed the next year by the pioneering work of George Melchers, George Labib and Itaru Takebe which showed that whole plants could be regenerated from tobacco leaf protoplasts (Takebe et al., 1971). The stage was set for what was then described as 'The New Botany' (Cocking, 1989). There were, however, some ongoing frustrations and delays in trying to imitate in cereals the plant regeneration from leaf protoplasts that had been achieved in tobacco. There was no difficulty in isolating cereal leaf protoplasts using our mixture of cellulase and pectinase. Unfortunately, however, whilst leaf protoplasts of wheat and barley would readily re-synthesize a wall, they would not undergo sustained division. I remember well a visit to Nottingham in the early 1970s by F. C. Steward who, with characteristic prescience, told us that chloroplast-containing protoplasts, at least in the cereals, were the worst starting material for cell culture. During his visit we demonstrated our method, using enzymes rich in β-1,3-glucanase, for the isolation of protoplasts from pollen tetrads (Bhojwani and Cocking, 1972) and within 5 min he was able to see them being released. Again, however, they did not undergo sustained division, but nevertheless 10 years later we were able to demonstrate their use in the production of gametosomatic hybrid plants by protoplast fusion (Pirrie and Power, 1986). The initial success in getting leaf protoplasts of tobacco and petunia to regenerate a cell wall and then undergo sustained division to form callus, with subsequent organogenesis to form roots and shoots, had pointed to the wrong path for cereal research. The right path arose from the foresight and perseverance of Indra and Vimla Vasil working with protoplasts isolated from suspension cultures derived from callus of pearl millet immature embryos. At the 5th International Symposium on Microbial and Plant Protoplasts held in Szeged, Hungary, in 1979, they reported their division to form embryoids and subsequently whole plants (Vasil and Vasil, 1980, 1992). At the time many researchers, including myself, doubted whether this approach would be better than the approach pioneered by Melchers and Takebe using protoplasts isolated directly from the plant, and whether the use of suspension cultures for protoplast isolation would be applicable to the World's major cereals, such as rice, wheat and maize (Cocking, 1979). We were, however, to be proved wrong. Indeed, working with rice protoplasts in an initiative supported by the Rockefeller Foundation, we were successful in regenerating whole plants from rice protoplasts, isolated not from leaves, but from cell suspension cultures (Abdullah et al., 1986).

Ongoing successes in basic studies on protoplast fusion and the selection of somatic hybrids leading to the development of the general area of somatic hybridization have been recounted in detail elsewhere (Evans and Cocking, 1978) and included the new opportunities provided for the diversification of cytoplasm (Kumar and Cocking, 1987), which is finding practical applications in the transfer of cytoplasmically based male sterility with potential application in hybrid rice production (Blackhall et al., 1998). This even extended to the fusion of plant protoplasts with animal cells; heterokaryons were produced synthesizing cellulose and haemoglobin (Salhani et al., 1985). I remember that when I showed Henry Harris a photograph of root meristem-derived protoplasts he said that they looked just like animal cells! Currently, there is a resurgence of interest in the fusion of protoplasts to produce somatic hybrid plants, and the realization that it may be possible thereby to avoid some of the problems of genetic instability of transgenic plants. This arises from the recent study of homologous pairing and recombination in backcross derivatives of tomato somatic hybrids (*Lycopersicon esculentum* (+) *L. peruvianum*). It was shown that interspecific somatic hybrids with imbalanced parental genomes can, following a first backcross, lead directly to the recovery of fertile allodiploid progeny coupled with the introgression of foreign chromosomes. Such backcross progeny derived from complex somatic hybrid germplasms provides precisely the spectrum of allodiploid individuals required in conventional breeding strategies (Parokonny et al., 1997). Moreover, we have also shown that *in situ* hybridization of chromosomes in intergeneric somatic hybrids can begin to provide new insights into the introgression of genes in major crops such as rice (Jelodar et al., 1999).

It is interesting to trace the development of my interests in the novel uses of protoplasts in plant biology, particularly in relation to the development of novel nitrogen-fixing symbiotic interactions between nitrogen-fixing bacteria and plants. To my pleasant surprise, in the early 1970s I was offered research funding by Arnold Spicer, then research director of the Lord Rank Research Centre, to investigate any novel aspect of protoplast biology! I chose to study the uptake of rhizobia by protoplasts. Although this was basically successful and led to a significant publication (Davey and Cocking, 1972), the vision of the regeneration of plants from protoplasts, which contained rhizobia and other diazotrophic bacteria and blue-green algae, was not realized. Nevertheless, failure in this respect did not deter me and a different approach involving the isolation of nodule protoplasts containing rhizobial bacteroids (Davey et al., 1973) and their fusion with nonlegume leaf protoplasts was explored, but again no regenerated nonlegume plants with intracellular rhizobia were obtained.

A totally new approach and another major 'turning point' arose indirectly from my decision in the early 1980s to investigate the possibility of the direct interaction of foreign DNA with plants. The objective was to eliminate the need to isolate protoplasts, subject them to the various manipulations required for interaction with foreign DNA and then to spend time regenerating whole plants from the modified protoplasts. After several years effort, I was able to describe an enzymatic procedure for the degradation, within a few minutes, of the cell wall at the apices of root hairs from a wide range of crop species which exposed the plasma membrane with partial protoplast release, whilst maintaining the functional integrity of the plant (Cocking, 1985). This exposure of the plasma membrane not only provided a point of entry for bacteria, such as rhizobia, into the plant but also an ability to isolate root hair protoplasts which were

shown to express their totipotency (Rasheed et al., 1990). Although such enzymatic treatment of legume root hairs was shown to remove a barrier to rhizobial-host specificity, and the formation of nodule-like structures on rice and other nonlegume crops was detected following inoculation with rhizobia, development of nitrogen-fixing nodules on nonlegume crops did not result. However, this 'turning point' was fortified by my being asked to organize a Rockefeller Foundation Discussion Meeting with Ivan Kennedy from the University of Sydney on *Biological Nitrogen Fixation: The Global Challenge and Future Needs* (Kennedy and Cocking, 1997). I returned after nearly 30 yr to the Villa Serbelloni. From amongst the wide-ranging discussions the importance of trying to establish diazotrophs, such as rhizobia, within the root systems of the world's major cereals was highlighted. This would provide an experimental system to investigate the extent to which endophytic rhizobia in, for instance, the xylem might be able to fix nitrogen of benefit to the plant. Rhizobia such as *Azorhizobium caulinodans* appear to secrete their own pectinases and cellulases to facilitate their entry into the root system by crack entry; there is no need to add extra cell-wall-degrading enzymes. Encouragingly, we have recently shown that the xylem of rice can be colonized by *A. caulinodans* (Gopalaswamy et al., 2000).

As in all scientific ventures, I have been helped and inspired by numerous colleagues both nationally and internationally on what has been a voyage of discovery, with a different research crew on board at various stages of the journey and at the various turning points. Encouragement came from my election to Fellowship of the Royal Society in 1983, Academia Europaea in 1993 and Foreign Membership of the Hungarian Academy of Sciences in 1995. The voyage continues, enabling me to say again, as I said in 1978 (Evans and Cocking, 1978), but now with even greater conviction, that there is little doubt that work on isolated protoplasts has become established as one of the most important fields in plant cell and tissue culture, and that protoplast technology is not only contributing very substantially to our understanding of the fundamental properties of plant cells but is also making a considerable contribution to applied plant biology. I feel that my studies on plant protoplasts well illustrate the viewpoint of Peter Medawar that 'Science at all levels of endeavour is a passionate enterprise and the pursuit of natural knowledge a sortie into the unknown'.

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