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IMMOBILISATION AND MECHANICAL SUPPORT OF INDIVIDUAL PROTOPLASTS

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Summary

Mesophyl cell protoplasts of *Vicia faba* were suspended in a solution consisting of 10% sodium alginate and 0.4 M mannitol. The protoplasts could be immobilized by cross-linking the alginate in the presence of 100 mM $CaCl_2$. Changes in the osmolarity of the external medium led to reversible shrinkage and swelling of the entrapped protoplasts. It was demonstrated by using the pressure probe technique that a pressure gradient (cell turgor pressure) of several 100 mbar is built up when the immobilized cells were transferred to hypotonic solution. By complexing the Ca^{2+} in the alginate matrix with sodium citrate buffer the protoplasts could be released from the matrix. No morphological change or alteration of the membrane permeability of the immobilized protoplasts was observed after a storage period of up to 14 days at 4°C in the matrix.

The regeneration of plants from enzymatically isolated protoplasts has aroused a great deal of interest because of the possible use of protoplast cultures for trapping new genetic material in isolated cells without walls. This technique could be used, for example, to produce cereals with improved properties for agriculture. Techniques for the transfer and entrapment of DNA in protoplasts are described in the literature [1—4]. Although the ability of isolated protoplasts to regenerate a complete plant has been demonstrated in a number of species [5], protoplasts isolated from various monocotyle-

donous species and cultured under a variety of experimental conditions in vitro have shown, at most, a limited ability to divide and form a callus [6]. The formation of a cell wall is an important step in cell division and a prerequisite for the restoration of the hydrostatic pressure difference between the external medium and the cell interior (i.e., the cell turgor pressure). There is much evidence [7,8] that cell turgor pressure plays a crucial role in cell division in intact plants, in cell enlargement and in growth, and that pressure has to exceed a certain level in order to initiate these processes. Moreover, cell turgor can directly affect membrane transport and the electrical membrane properties at the cellular level [9,10]. The experimental observation that the membrane potential of protoplasts isolated from different species assumes positive values, as opposed to the negative value observed in the corresponding intact plant cells, may be due to zero turgor pressure in protoplasts [11,12].

One possible way of assessing the significance of cell turgor in membrane processes and in cell wall formation is described in this communication, in which protoplasts are stabilized by immobilization in a polymeric matrix, the mechanical properties of which can be varied by using different reactants and by changing the degree of cross-linkage. Entrapment of micro-organisms in a polymeric matrix has demonstrated that the cellular functions and the membrane integrity are apparently not influenced by the process of entrapment [13–15]. On the contrary, micro-organisms have been seen to survive for longer under those conditions than under comparable conditions in suspension cultures. The technique described here of immobilization of mesophyll cell protoplasts of *Vicia faba* in a polymeric matrix while apparently retaining their cellular functions can also be applied to other wall-less cells, such as erythrocytes, and to lipid vesicles. It would thus be possible, in principle, to study the structure and function of both artificial and biological membranes in the presence of pressure gradients.

The procedure of ionic network formation was used for immobilization [16]. Alginate (Manucol LD, Alginate Industries Ltd., London) is cross-linked in the presence of high concentrations of Ca^{2+}. The addition of a Ca-complexing agent leads to the dissolution of the matrix, releasing the trapped protoplasts which may then be examined for any change in their cellular and membrane functions. Since a high concentration of Ca^{2+} (greater than 5 mM) leads to changes in the cell membrane, the experiments have to be carried out in such a way that the cross-linking of the alginate (with its concomitant binding of Ca^{2+}) proceeds rapidly enough to prevent a prolonged exposure of the cell membranes to the increased Ca concentration.

In detail, the procedure was as follows. Plants of *V. faba* were maintained in a culture chamber and grown in a standard soil in a 12 h light and dark regime. The daytime temperature was 20°C with a relative humidity of 75% and light intensity of 15 000 lux (Osram, L 65W/20 R, hellweiss). At night the temperature was maintained at 17°C with a relative humidity of 85%. The leaves of 3-week-old plants were immersed in water for 30 min after which the lower epidermis was stripped off. The leaves were cut into segments of about 5 mm^2 which were exposed to a solution containing 4% cellulysin (supplied by Calbiochem, San Diego, CA, U.S.A.) with 0.4 M mannitol and and 1mM $CaCl_2$, pH 5.3, and shaken occasionally over a period of 1–3 h at

room temperature [17]. After filtration through a sieve with a mesh size of 56 μm the cells were washed in 0.4 M mannitol and 1 mM $CaCl_2$, centrifuged at 50 × *g* for 3 min and collected in a solution as described above. In order to immobilize the protoplasts, 1 vol. of packed cells was carefully mixed with 9 vols. of a 10% solution of sodium alginate in 0.4 M mannitol (pH 6.2). The mixture was spread into a slide and covered for 1 min with a solution containing 0.4 M mannitol and 100 mM $CaCl_2$. In order to achieve complete cross-linking of the matrix, and to preserve the immobilized protoplasts, a 0.6 M mannitol solution containing 5 mM $CaCl_2$ was subsequently used (in some cases with the addition of 1% penicillin/streptomycin; so as to prevent the growth of bacteria during preparation and storage). As shown in Fig. 1, a large proportion of mesophyll protoplasts of *V. faba* can be immobilized in cross-linked calcium alginate under these conditions. A particularly high rate of survival can be achieved in immobilized cells if the sodium alginate solution is slightly hypotonic rather than hypertonic. No morphological change in the structure of the cells could be detected over a storage period up to 14 days

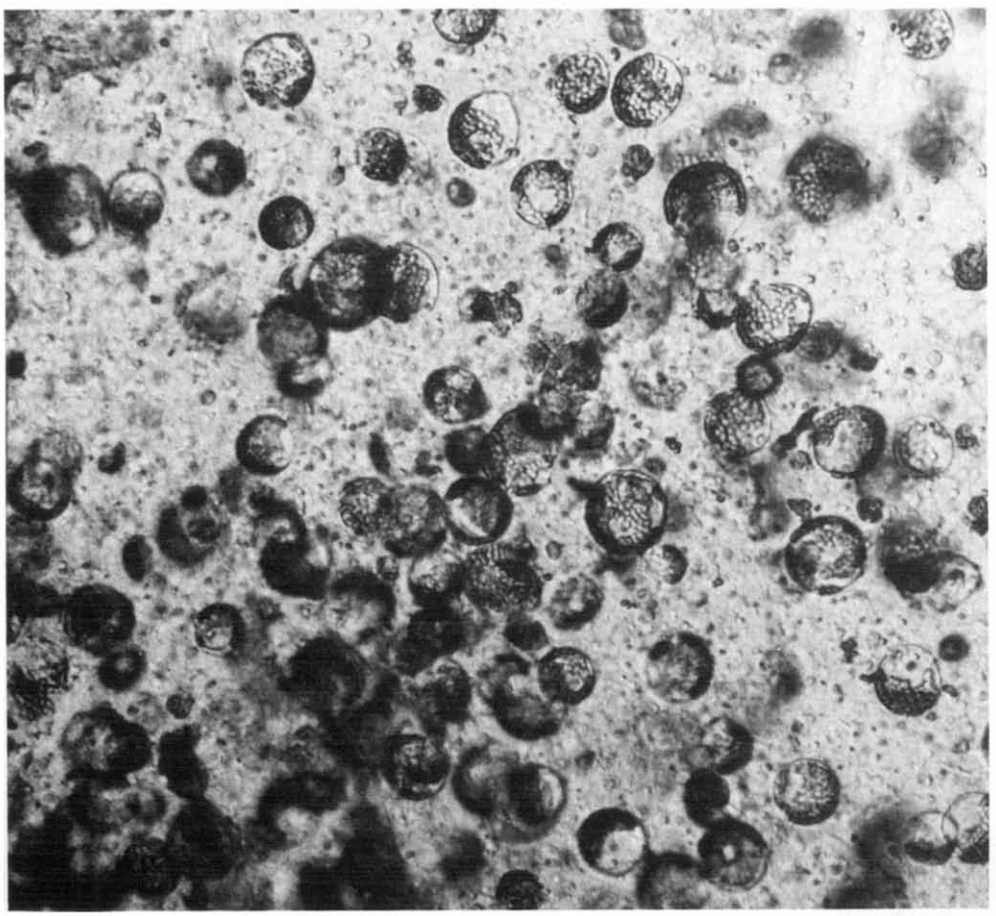

Fig. 1. Mesophyll cell protoplasts of *Vicia faba* immobilized in cross-linked calcium alginate. Protoplasts were carefully mixed with a 10% solution of sodium alginate in 0.4 M mannitol and the mixture was cross-linked with Ca^{2+} on a slide, for further explanation see text. Scale: 1 cm = 35 μm.

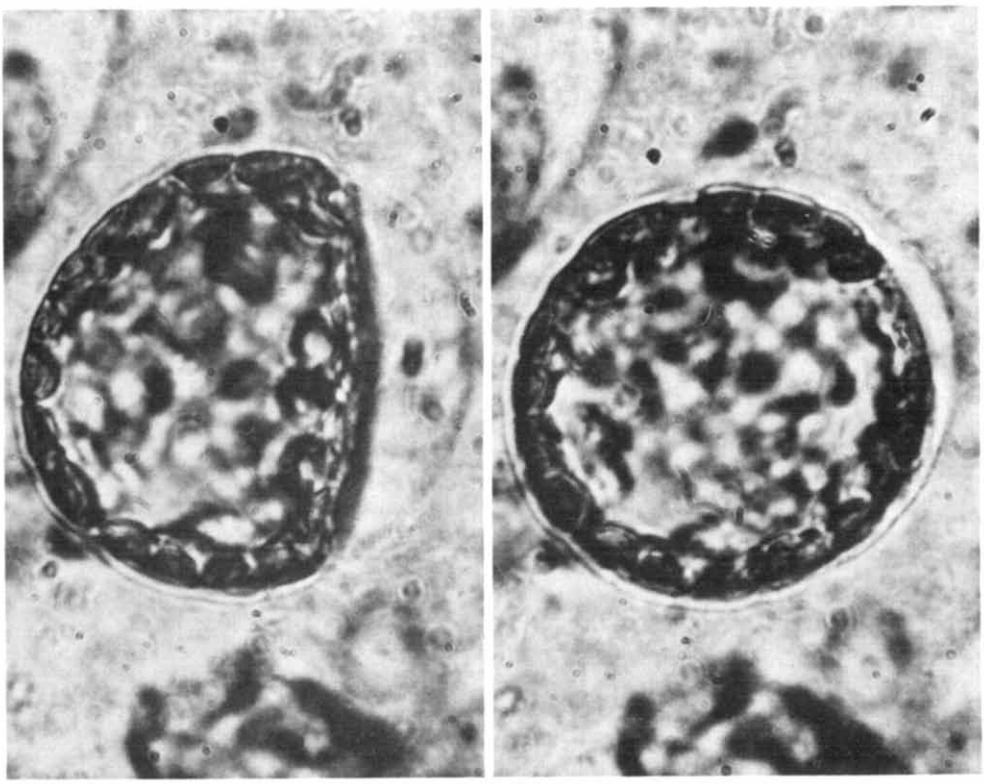

Fig. 2. Reversible shrinking and swelling of a mesophyll cell protoplast of *Vicia faba* immobilized in calcium alginate. The immobilized cells stored in 0.6 M mannitol solution were transferred in a solution consisting of 0.7 M mannitol and 5 mM $CaCl_2$ for 10 min (a). Subsequent swelling of the same protoplast occurred by reducing the mannitol concentration to 0.5 M (b). Scale: 1 cm = 7 μm.

at 4°C. Since the cross-linked matrix is permeable to water the immobilized protoplasts react to changes in the osmolarity of the external solution. Fig. 2 demonstrates the reversible shrinkage and swelling of a protoplast after the matrix containing the cells had been transferred to a 0.7 M mannitol solution and subsequently to a solution of 0.5 M (both containing 5 mM $CaCl_2$). It was evident that the plasmalemma had become partly detached from the alginate coat. Volume changes in response to external osmotic stress were observed even after the protoplasts had been maintained in the polymeric matrix at 4°C for 14 days. Subsequent reduction of the external osmolarity resulted in the build-up of a pressure gradient (i.e., cell turgor pressure). This could be shown directly with the pressure probe. The principle of this technique has already been described in a number of previous publications (for reviews see Refs. 18—20). Briefly, after the introduction of a microcapillary (tip diameter about 3 μm) into the cell, the pressure is transmitted through oil to a pressure transducer which transforms the pressure signals into proportional voltage signals. The oil/cell sap boundary in the very tip of the capillary is regulated electronically to a given position so that any leakages in the pressure-measuring device or temperature fluctuations, etc., are compensated for. The pressure transducer measures pressure accurately above 0.03–0.05 bar. The mobility

of the meniscus in the tip of the capillary may be used as a tool to test whether the capillary tip is blocked with cellular material or with cross-linked alginate. Such a blockage would lead to erroneous measurements (this aspect is described elsewhere [18—20]. It was possible to demonstrate unequivocally that the capillary tip was not blocked after it had been introduced into the cells which were immobilized closely to the surface of the polymeric matrix. This means that it should be possible in future to measure both the potential and the resistance in mechanically stabilized protoplasts with micro-electrodes. The recorded pressure values in different experiments varied between 100 and 300 mbar after the addition of water to the external solution. As the pressure probe measures within an accuracy of ± 20 mbar [20] it could be clearly shown that the protoplasts had built up a cell turgor pressure in hypotonic medium whereas no pressure was found in 0.6 M mannitol solution. Cell turgor pressure in immobilized protoplasts caused by the addition of water to the external medium was measured both in freshly prepared and immobilized cells and after storage of the gels for several days. This finding suggests that the mechanical support of the alginate and not cell wall formation (see below) is responsible for the appearance of turgor pressure.

By complexing the Ca^{2+} in the alginate matrix with sodium citrate buffer the protoplasts may be released from the matrix. For this purpose the polymeric matrix was slightly shaken in a 0.8 M mannitol solution with 20 mM sodium citrate at pH 7.4. The alginate takes about 1 h to dissolve. The solution was centrifuged and the pellet washed in a large volume of 0.8 M mannitol solution with 5 mM $CaCl_2$. Morphologically the cells exhibited no change when compared with freshly isolated mesophyll cell protoplasts (Fig. 3). They are stained with neutral red, a vital dye, and react to osmotic stress with corresponding changes in cell volume. Staining with the cellulose fluorescent brightener, calcofluor ST, showed that no cell wall had formed during a storage period of up to 7 days.

The results presented here demonstrate that it is possible to immobilize wall-less cells in a polymeric matrix and to set up pressure gradients between the cells and their environments. None of the experiments so far have given any indication that there are any irreversible changes in the cells themselves or in the cell membranes during the process of polymerization and storage. It will certainly be necessary to perform enzyme tests in order to arrive at some final conclusion about the biochemical activity of the cell.

However, even at this early stage, it is evident that the entrapment technique described here provides a new tool to help in studies of membrane transport and cellular functions in cells without walls in the presence of pressure gradients and under defined experimental conditions. Experiments of this kind not only help to elucidate the processes involved in the regeneration of a complete plant from a protoplast, but also provide a method of storing and even transporting cells, which would otherwise be unstable in suspension culture, over long periods of time. This is particularly true for guard cell protoplasts, certain plant cell cultures (such as *Chenopodium rubrum*) and for a number of animal cells, e.g., transformed cells.

The turgor pressure measurements in immobilized protoplasts presented here were performed by Dipl. Biol. K.-H. Büchner. We wish to thank Dipl.

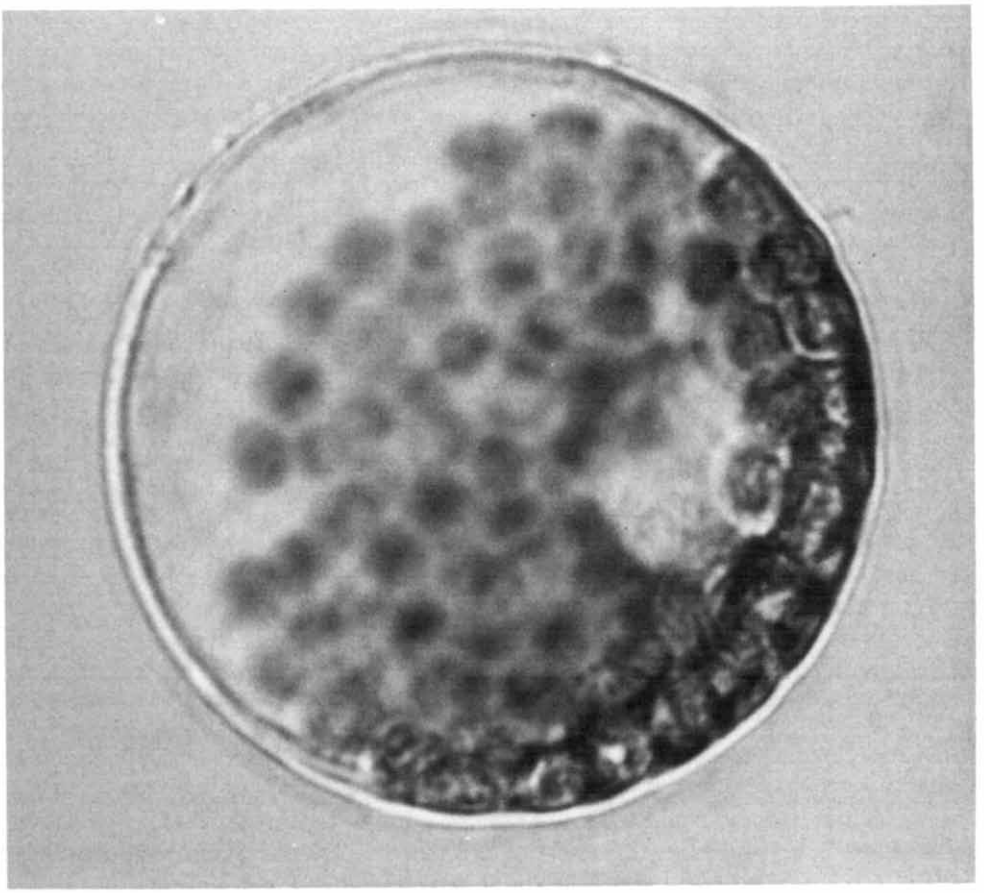

Fig. 3. A mesophyll cell protoplast of *Vicia faba* stored for 4 days in the alginate matrix and released from the polymerized network by addition of a sodium citrate buffer (20 mM sodium citrate, 0.8 M mannitol, pH 7.4). The released protoplasts were reincubated in a solution consisting of 0.8 M mannitol and 5 mM $CaCl_2$. Scale: 1 cm = 5 μm.

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