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SHORT COMMUNICATION

A novel method of measuring volume changes of mesophyll cell protoplasts and the effect of mercuric chloride on their osmotically-induced swelling

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Abstract

A quick and accurate method of monitoring changes of volume of mesophyll cell protoplasts (MCP) of pea was devised using the difference in absorbance of the protoplasts at 440 nm and 750 nm (OD 440–750); when protoplasts expanded in response to changing the external medium from 0.4 M sorbitol to 0.3 M sorbitol OD 440–750 values increased and, conversely, when protoplasts were transferred from 0.4 to 0.5 M sorbitol, protoplasts contracted. The kinetics of expansion or contraction of the protoplasts could also be monitored using this method and the half-time for water exchange for expanding protoplasts (about 10 s) was slightly higher than that for contracting protoplasts. A study of the effects of the water channel blocker, mercuric chloride, on swelling protoplasts showed that 500 µM irreversibly damaged protoplasts, 5–10 µM had a negligible inhibitory effect on swelling while 100 µM had a large inhibitory effect often completely inhibiting swelling. A preliminary study indicated that mercaptoethanol reversed the inhibitory effect of mercuric chloride on protoplast swelling.

Key words: Aquaporins, endocytosis, exocytosis, osmosis, protoplasts.

Introduction

Protoplasts have not been extensively used to study water permeabilities of membranes even though they appear ideal subjects since volume changes in response to osmotic gradients readily occur in the absence of cell walls. Possible reasons for this are that protoplasts are delicate objects and it is not always easy to prepare healthy, viable ones, and also it is not easy to measure the kinetics of their changing dimensions in response to an osmotic gradient. Recent studies used vesicles prepared from root cells of wheat (Niemietz and Tyerman, 1997) or from suspension-cultured tobacco cells (Maurel *et al*., 1997) and indirectly measured changes in volume by monitoring light scattering of a suspension of vesicles with stopped-flow spectrofluorimetry. Such a system is difficult to use with protoplasts since they sediment relatively quickly. In this study, however, a method of indirectly measuring volume changes of protoplasts is described and the effects of mercuric chloride and mercaptoethanol on the volume changes induced by osmotic gradients are reported in order to investigate the involvement of water channels in such phenomena. Mercury ions are reported to inhibit water movement through water channels (aquaporins) while sulphydryl reagents, such as mercaptoethanol, are reported to reverse the inhibition (Steudle and Henzler, 1995).

Materials and methods

Plant material and protoplast preparation

Mesophyll cell protoplasts (MCP) of pea (*Pisum sativum* cv. Bonneville) were used to monitor volume changes and optical density changes in response to varying sorbitol concentrations. Plants were grown as described in Saradadevi *et al*. (1996) and MCP isolated from leaves according to the method of Saradadevi and Raghavendra (1992).

Correlation of MCP volume with OD 440–750 in response to a range of sorbitol concentrations

The method of monitoring volume changes of MCP was based on an observation by Saradadevi *et al*. (1996) who found that

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the absorbance of a sample of protoplasts at 440 nm corrected for turbidity at 750 nm could be used as a measure of their average volume. Studies in this report are described which develop and standardize this method for following volume changes of protoplasts.

The effect of a range of sorbitol concentrations (between 1 M and 0.1 M) on the absorbance difference between wavelength readings at 440 and 750 nm (OD 440–750 nm) of an MCP suspension was measured with a spectrophotometer (Shiadzu UV160A) in the following way. An MCP sample, of known chlorophyll concentration (usually between 10 and 20 μg chlorophyll cm^{-3}, equivalent to between 0.5×10^6 and 1×10^6 MCP) was added directly to a cuvette containing 1 cm^3 sorbitol. The cuvette was inverted twice to mix the MCP sample and the absorbance difference measured 2 min later after again inverting the cuvette twice.

Volume changes of MCP were monitored in the following way. A 10 μl sample was injected into 1 cm^3 of each sorbitol concentration and after 2 min the diameters of 50 MCP were measured using a Wolfe microscope at ×400 magnification with a calibrated eye-piece. An average volume was calculated from the mean diameter of the 50 MCP.

Effect of the population density on OD 440–750

In order to find the protoplast concentration which gave maximum absorbance differences over a range of sorbitol concentrations (0.3–0.6 M), samples of MCP equivalent to 5, 10, 20, 30, 40, and 50 μg chlorophyll, were used.

Kinetics of swelling and contraction of MCP as gauged by OD 440–750

Mesophyll cell protoplasts were isolated in 0.4 M sorbitol and a sample equivalent to 20 μg chlorophyll injected into 1 cm^3 0.3 M or 1 cm^3 0.5 M sorbitol. The OD 440–750 was measured at various time intervals up to 15 min. At each time interval the cuvette containing the protoplasts was inverted twice, immediately before measurements were made.

Effects of mercuric chloride and of mercaptoethanol on volume changes of MCP

Mesophyll cell protoplasts were isolated in 0.4 M sorbitol and a sample equivalent to 20 μg chlorophyll injected into 1 cm^3 0.3 M sorbitol containing different concentrations of mercuric chloride (5, 10, 50, 100, 500 μM). At various time intervals the OD 440–750 was measured using the same procedure as described above.

In another series of experiments a sample of MCP were preincubated for 10 min in 0.4 M sorbitol containing mercuric chloride of different concentrations (5–500 μM). Then a sample equivalent to 20 μg chlorophyll was injected into 1 cm^3 0.3 M sorbitol and the OD 440–750 monitored over time. Using 50 μM mercuric chloride, after an equilibrium OD 440–750 value was obtained, mercaptoethanol (final concentration, 70 mM) was injected into the cuvette containing the MCP and the OD difference continued to be measured.

Chlorophyll estimation

Chlorophyll concentrations of the purified MCP samples were determined, after extraction of a subsample in 80% acetone, according to the method of Arnon (1949).

Results

The effect of a range of sorbitol concentrations (0.25–0.55 M) on MCP volume and on absorption difference (OD 440–750 nm) was first ascertained (Fig. 1). Both MCP volume and OD 440–750 decreased as sorbitol concentration increased in a linear relationship, with the exception of volume change in 0.25 M sorbitol. When volume changes were monitored over a wider range of sorbitol concentration protoplast bursting occurred in 0.1 M while in 0.8 M and higher sorbitol concentrations protoplasts were frequently not spherical. Figure 2 shows the relationship between OD 440–750 and protoplast volume. An excellent fit of a line ($r^2 = 0.975$) was achieved using Cricket Graph and polynomial regression. A sigmoidal relationship was obtained with each of the extreme points diverging from a straight line. This may again be due to some bursting of the largest protoplasts at the low sorbitol concentration (0.25 M) and lack of sphericity of protoplasts at the high concentration (0.55 M).

To optimize the range of OD 440–750 values over a range of sorbitol concentrations, MCP populations (equivalent to 5, 10, 20, 30, 40, and 50 μg chlorophyll cm^{-3}) were used (Fig. 3). Over a range of sorbitol concentrations between 0.3 and 0.6 M the widest range of OD 440–750 values was obtained at about 20 μg cm^{-3} chlorophyll and this range was maintained above this chlorophyll concentration. Therefore concentrations of around 20 μg cm^{-3} chlorophyll were used in future experiments.

The kinetics of swelling and contraction of the MCP was monitored next (Fig. 4). Three replicate experiments

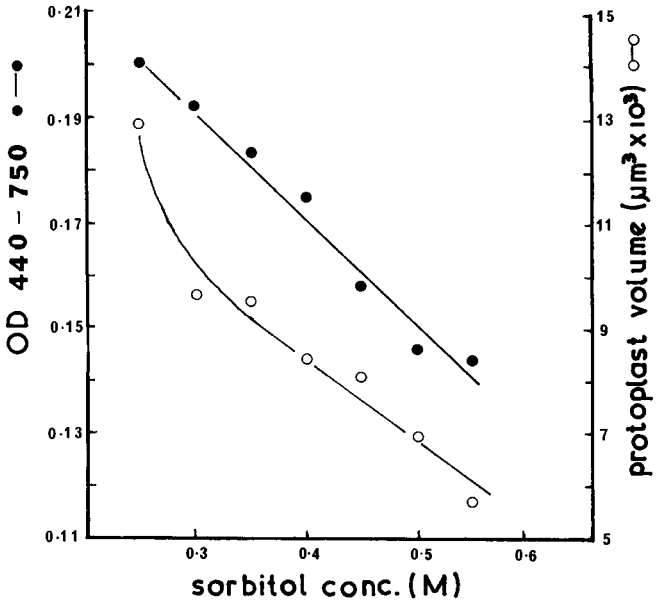

Fig. 1. The effect of sorbitol concentration on protoplast volume and absorption difference, 440–750 nm. The protoplast population was equivalent to 10 μg chlorophyll cm^{-3}.

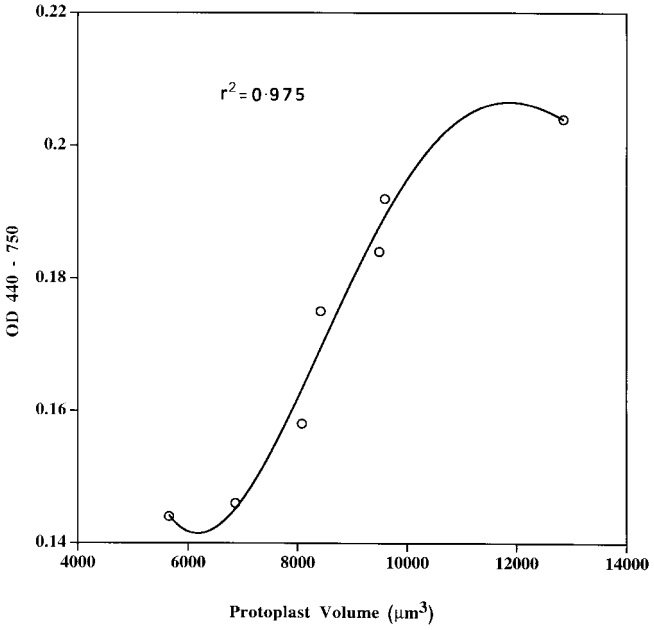

Fig. 2. The relationship between OD 440–750 and protoplast volume. The line was fitted using a 4th order polynomial regression and Cricket Graph, and values presented in Fig. 1 were used to make the plot; $r^2 = 0.975$.

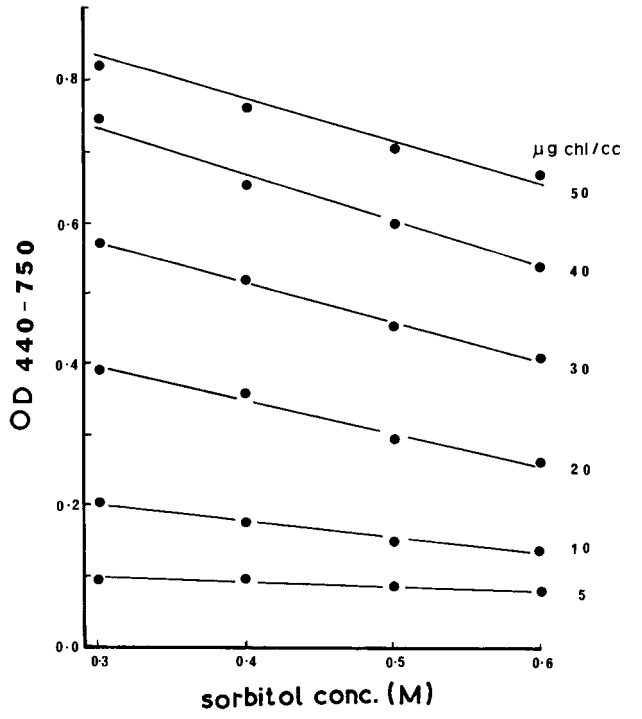

Fig. 3. The effect of sorbitol concentration on absorption difference, 440–750 nm, at different chlorophyll (protoplast populations) concentrations in the cuvette.

showed similar swelling responses of the MCP when they were transferred from 0.4 to 0.3 M sorbitol. Maximum volumes were achieved after about 2 min giving half-times for water exchange of about 10 s. When protoplasts were transferred from 0.4 to 0.5 M sorbitol to contract them three replicate experiments showed similar kinetics with maximum volumes achieved after 1–2 min though there was some variation in their extent of contraction, and volumes slowly increased thereafter.

Because swelling protoplasts gave consistent and repeatable results further experiments concentrated on this aspect of volume changes. Figure 5 shows the effect of a range of concentrations of mercuric chloride on protoplast swelling. Protoplasts were isolated in 0.4 M sorbitol and then incubated in 0.3 M sorbitol containing different mercuric chloride concentrations and volume changes compared with control values in which protoplasts were either maintained in 0.4 M sorbitol or placed in 0.3 M sorbitol. As mercuric chloride concentrations increased from 5 μM to 500 μM the final protoplast volume decreased: 5 μM and 10 μM mercuric chloride concentrations allowed considerable swelling but 100 μM mercuric chloride completely prevented swelling relative to volumes of protoplasts maintained in 0.4 M sorbitol; 500 μM mercuric chloride caused protoplasts to shrink well below volumes for protoplasts maintained in 0.4 M sorbitol and inspection of them under a microscope indicated that they were damaged.

Characteristically, in the presence of mercuric chloride, the protoplasts initially swelled at similar rates (except in the 500 μM mercury treatment) before contracting back to more stable volumes at a level which was dependent on the concentration of mercury used. It was considered that such swelling kinetics may be due to volume changes of the protoplasts occurring before the effects of the mercury became active. Therefore experiments were conducted in which protoplasts were preincubated in 0.4 M sorbitol with mercuric chloride before placing them into 0.3 M sorbitol also containing appropriate mercuric chloride concentrations.

Figure 6 shows the results of preincubating protoplasts in the different mercury concentrations. The general pattern of effects was similar to that observed when preincubation in mercury was not carried out (Fig. 5) but the contraction of protoplasts after initial volume increases was much less evident and prominent only in 500 μM mercuric chloride. 500 μM mercuric chloride ultimately damaged protoplasts and volumes dropped well below control values obtained when protoplasts were maintained in 0.4 M sorbitol: final volumes in 100 μM mercury were about the same as for protoplasts maintained in 0.4 M sorbitol while 50 μM and 10 μM mercury slightly inhibited and had no detectable effect on protoplast swelling, respectively.

Discussion

Results indicated that values of OD 440–750 was a relatively accurate, quick and convenient measure of protoplast volume, particularly when swelling was being

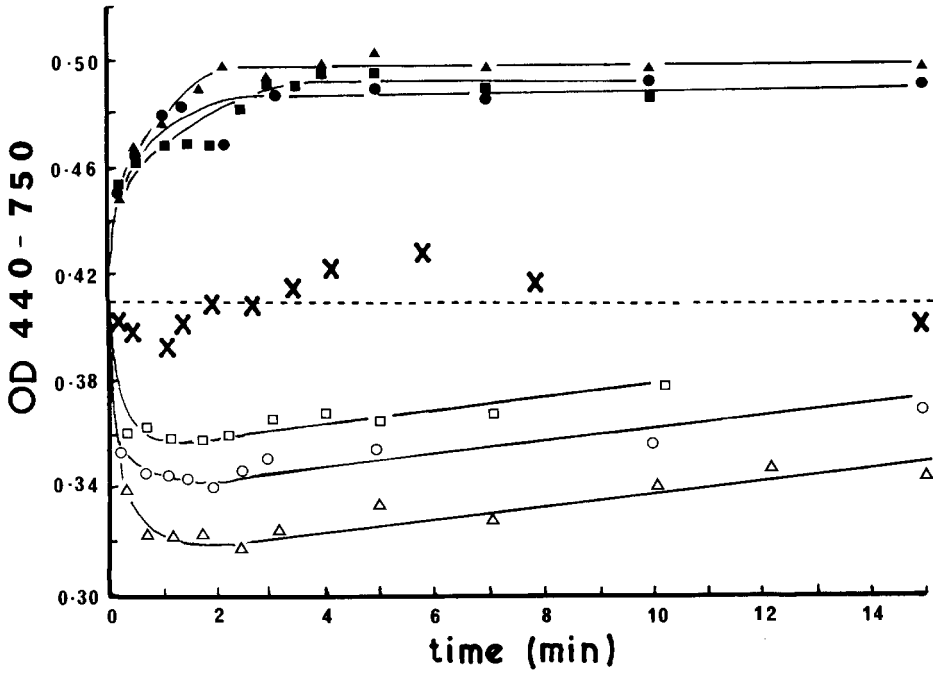

Fig. 4. The kinetics of protoplast swelling or contraction (equivalent to changes of OD 440–750 nm) in response to changing the suspension medium from 0.4 M to 0.3 M or to 0.5 M sorbitol. Three replicate traces are presented for swelling (●, ■, ▲) and contraction (○, □, △) and a dashed line (x---x) represents protoplast volumes maintained in 0.4 M sorbitol. All lines are fitted by eye. The protoplast population was equivalent to 20 μg chlorophyll cm^{-3}.

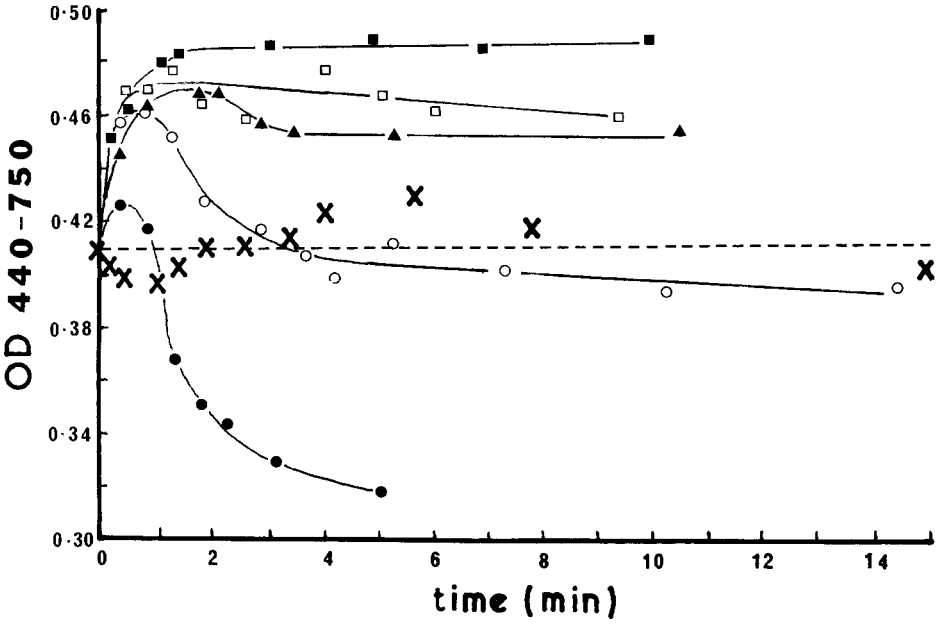

Fig. 5. The effect of different mercuric chloride chloride concentrations on swelling of protoplasts induced by changing the suspension medium from 0.4 M to 0.3 M sorbitol. The 0.3 M sorbitol contained the indicated mercury concentration. Key: (x---x), protoplasts maintained in 0.4 M sorbitol; (■), zero mercury; (□) 5 μM mercury; (▲) 10 μM mercury; (○), 100 μM mercury; (●) 500 μM mercury. Lines were fitted by eye. The protoplast population was equivalent to 20 μg chlorophyll cm^{-3}.

monitored (Figs 1–4). Consistent and repeatable values of protoplast volumes could be obtained when swelling was monitored, but there was some variation in protoplast volumes during contraction experiments and stable volumes were not achieved (Fig. 4). Thus, OD 440–750 values indicated that during contraction experiments protoplasts contracted to a minimum volume before starting to swell slowly and this continued for up to at

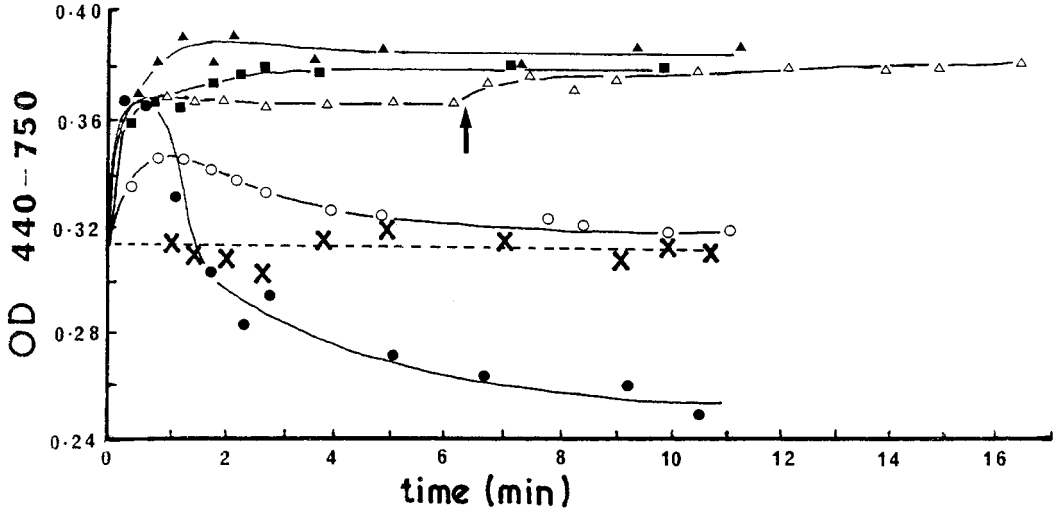

Fig. 6. The effect of different mercuric chloride concentrations on swelling of protoplasts induced by changing the suspension medium from 0.4 M to 0.3 M sorbitol. Protoplasts were preincubated for 30 min in 0.4 M sorbitol containing different mercury concentrations before placing in 0.3 M sorbitol containing equivalent mercury concentrations as in the 0.4 M sorbitol. Key: (x---x), protoplasts maintained in 0.4 M sorbitol; (■), zero mercury; (▲), 10 μM mercury; (○), 100 μM mercury; (●), 500 μM mercury; (△), protoplasts exposed to 50 μM mercury and treated with 70 mM mercaptoethanol (ME) at the point indicated by the arrow. Lines were fitted by eye. The protoplast population was equivalent to 18 μg chlorophyll cm^{-3}.

least 15 min (the duration of experiments). The re-swelling of the protoplasts is probably due to increased internal osmotic pressure perhaps because of uptake of sorbitol, the external osmoticum. If this is the case, the reasons why the plasma membrane becomes permeable to sorbitol are not clear and demand further attention in the future.

The technique used for monitoring volume changes also enables some studies of the kinetics of protoplast swelling and contraction to be followed (Fig. 4). However, it was not feasible to obtain values of protoplast volume within the first 15 s due to initial manipulations of the sample, making calculation of rate constants less accurate, and therefore only values of half-times for water exchange are quoted. The half-time for osmotic water exchange of cells and protoplasts is typically much less than 1 min and usually less than 30 s (Tyerman and Steudle, 1982; Dowgert *et al.*, 1987). Values for protoplasts obtained in this study were relatively short being about 10 s for expanding protoplasts and less for contracting protoplasts (Fig. 4). A reason for the lower half-times of water exchange of protoplasts relative to cells may be related to the effects of unstirred layers and limited diffusion of osmoticum in the cell walls (Maurel, 1997). If the differences in half-times for swelling and contracting protoplasts are genuine it is interesting to speculate why this is so. One possibility is that insertion into (exocytosis) and extraction from (endocytosis) the plasma membrane of membrane material, which may be associated with expansion and contraction of protoplasts, respectively, is related to the different values. This aspect is discussed in more detail below.

The water permeability of a protoplast is the sum of the permeabilities for the plasma membrane, cytoplasm and tonoplast. Virtually all reports indicate that the tonoplast membrane permeabilities for all substances measured are much greater than those for the plasma membrane. Thus, Dainty and Hope (1959) and Kiyosawa and Tazawa (1977) observed this feature in *Chara* cells; Url (1971) using onion bulb epidermal cells and plasmometric methods found that the tonoplast was more permeable to water than the plasma membrane. More recently, Maurel *et al.* (1997) using tonoplast and plasma membrane-enriched vesicles of suspension-cultured tobacco cells, and Niemietz and Tyerman (1997), using vesicles of plasma membrane and of endomembrane (including tonoplast) of wheat root, also concluded that the tonoplast-containing fractions were much more permeable to water than the plasma membrane fractions. If this can be universally applied to all higher plant cells then, in these experiments, using mesophyll cell protoplasts of *P. sativum*, the kinetics of water flux is being effectively observed at the plasma membrane since it has the bigger resistance to water flow.

The observation that protoplasts appeared more permeable to water when contracting rather than when expanding may be related to exocytotic and endocytotic processes that occur as the protoplasts swell or contract, in the following way. It is considered that membranes can only stretch by about 2–3% before they break (Wolf and Steponkus, 1983). In this study protoplast volumes increased by about 40% when they were changed from 0.5 M sorbitol to 0.3 M sorbitol. Thus, new membrane

material must be inserted into existing plasma membrane to accommodate swelling while, upon contraction, existing membrane must be absorbed to maintain protoplast sphericity. It is possible that membrane material inserted into the existing plasma membrane has a lower density of aquaporins than the original, a feature which would increase half-time exchanges for water and give different values of water permeabilities for protoplasts depending on whether they were contracting or swelling. Presumably investigators using vesicles to study water exchange are not creating large differences of membrane surface area with changing osmotic gradients otherwise they would burst.

In swelling experiments 100 μM mercuric chloride was most effective at inhibiting swelling; 500 μM mercury damaged protoplasts while 50 μM mercury inhibited swelling to only a small extent (Figs 5, 6). In some previous studies relatively high mercury concentrations have been used to investigate water conductivities. For example, Maggio and Joly (1995) found that 500 μM mercuric chloride caused a large and rapid reduction in pressure-induced root water flux in tomato and 60 mM mercaptoethanol largely reversed the inhibition. 500 μM mercury irreversibly damaged our protoplasts, but it is possible that when whole roots or tissues are used in experiments such high external concentrations are needed to bring about enough penetration of mercury to the centre of the tissues. Maurel *et al*. (1997), however, found that preincubation of tonoplast-enriched vesicles for 5 min in concentrations of up to 1 mM mercuric chloride were reversible. Usually, however, much lower concentrations of mercuric chloride than 500 μM are used in experiments whether tissues, cells, protoplasts or organelles are the subject of investigation (Hejnowicz and Sievers, 1996; Steudle and Henzler, 1995; Niemietz and Tyerman, 1997).

If water movement across the plasma membrane occurs via diffusion through aquaporins and the lipid bilayer then it would be expected that mercury (which blocks water movement through aquaporins) would slow down the rate of water movement and associated rates of protoplast volume change rather than effect absolute values of volume change. Figures 5 and 6, however, appear not to support this contention. It is possible, though, that equilibrium volumes of the protoplasts treated with mercury may take longer than 15 min to be attained which was the maximum time over which observations were usually made in this study. Moreover, at the higher mercury concentrations cell damage is occurring with concomitant solute leakage so that protoplast volumes equivalent to control values are not to be expected.

In a preliminary experiment the effect of mercaptoethanol on the inhibition of protoplast swelling by 50 μM mercuric chloride was studied (Fig. 6). Although 50 μM mercury inhibited the absolute amount of swelling to a small extent it was evident that 70 mM mercaptoethanol reversed the inhibition allowing protoplast volumes to reach control values. Future work will investigate this aspect in more detail.

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References

Arnon DI. 1949. Copper enzymes in isolated chloroplasts. polyphenol oxidase in *Beta vulgaris*. *Plant Physiology* **24,** 1–15.

Dainty J, Hope P. 1959. The water permeability of cells of *Chara australis* R. Br. *Australian Journal of Biological Science* **12,** 136–145.

Dowgert MF, Wolfe J, Steponkus PL. 1987. The mechanics of injury to isolated protoplasts following osmotic contraction and expansion. *Plant Physiology* **83,** 1001–1007.

Hejnowicz Z, Sievers A. 1996. Reversible closure of water channels in parenchymatic cells of sunflower hypocotyl depends on turgor status of the cells. *Journal of Plant Physiology* **147,** 516–520.

Kiyosawa K, Tazawa M. 1977. Hydraulic conductivity of tonoplast-free *Chara* cells. *Journal of Membrane Biology* **37,** 157–166.

Maggio A, Joly RJ. 1995. Effects of mercuric chloride on the hydraulic conductivity of tomato root systems. Evidence for a channel-mediated water pathway. *Plant Physiology* **109,** 331–335.

Maurel C. 1997. Aquaporins and water permeability of plant membranes. *Annual Review of Plant Physiology and Plant Molecular Biology* **48,** 399–429.

Maurel C, Tacnet C, Guclu J, Guern J, Ripoche P. 1997. Purified vesicles of tobacco cell vacuoles and plasma membranes exhibit dramatically different water permeabilities and water channel activity. *Proceedings of the National Academy of Sciences, USA* **94,** 7103–7108.

Niemietz CM, Tyerman SD. 1997. Characterization of water channels in wheat root membrane vesicles. *Plant Physiology* **115,** 561–567.

Saradadevi K, Padmasree K, Raghavendra AS. 1996. Correlation between the inhibition of photosynthesis and the decrease in area of detached leaf discs or volume/absorbance of protoplasts under osmotic stress in pea (*Pisum sativum*). *Physiologia Plantarum* **96,** 395–400.

Saradadevi K, Raghavendra AS. 1992. Dark respiration protects photosynthesis against photoinhibition in mesophyll protoplasts of pea (*Pisum sativum*). *Plant Physiology* **99,** 1232–1237.

Steudle E, Henzler T. 1995. Water channels in plants: do basic concepts of water transport change? *Journal of Experimental Botany* **46,** 1067–1076.

Tyerman SD, Steudle E. 1982. Comparison between osmotic and hydrostatic water flow in a higher plant cell: determination of hydraulic conductivities and reflection coefficients in isolated epidermis of *Tradescantia virginiana*. *Australian Journal of Plant Physiology* **9,** 461–479.

Url WG. 1971. The site of penetration resistance to water in plant protoplasts. *Protoplasma* **72,** 427–447.

Wolfe J, Steponkus PL. 1983. Mechanical properties of the plasma membrane of isolated protoplasts. *Plant Physiology* **71,** 276–285.