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Behaviour in Culture of Isolated Protoplasts from "Paul's Scarlet" Rose Suspension Culture Cells

R. S. Pearce and E. C. Cocking

Department of Botany, University of Nottingham, Nottingham, England

With 11 Figures

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Summary

Protoplasts were enzymatically isolated from "Paul's scarlet" rose suspension culture cells. They were cultured in medium similar to that used to culture the cells from which they were isolated with the addition of sucrose as an osmotic stabiliser. They were studied by light and electron microscopy and their changes in size and number per culture were recorded. Expansion was greater when the protoplasts were cultured in medium plus 12% sucrose than with 24% sucrose. Budding was observed. In medium plus 12% sucrose about 45% of the protoplasts divided but in medium plus 24% sucrose far fewer divided. Cytokinesis was abnormal: the phragmoplast disappeared soon after cytokinesis began and the cell plate became a groove and then a fibril-lined or filled tongue which progressed across the vacuole, unconnected by strands to other parts of the protoplast. The wall regenerated after several days culture in medium plus 12% sucrose fluoresced with calcofluor. The wall regenerated in medium with 24% sucrose fluoresced usually only after several weeks culture. Cytokinesis hastened formation of a wall fluorescing with calcofluor. In the electron microscope the wall was seen to contain fibrils and non-fibrillar material. The latter was the minor component in medium plus 12% sucrose but was usually the major component in medium plus 24% sucrose. The growth in plasmolysing and non-plasmolysing medium of the cells from which protoplasts are isolated was also studied.
It appears that loss of the wall alters the potential of protoplasts to expand and possibly also to regenerate a wall and to divide. Wall regeneration is initially linked with expansion and cytokinesis. Osmotic pressure of the external medium is also an important factor.

1. Introduction

Division of isolated protoplasts from several plants and cultures has been reported (Nagata and Takebe 1970, Kao *et al.* 1970, Grambow *et al.* 1972, Kameya and Uchimiya 1972, Potrykus and Durand 1972). The culture of protoplasts enzymatically isolated from leaves of *Nicotiana tabacum* L. cv., Xanthi nc has been extensively studied (Nagata and Takebe 1970, 1971, Takebe *et al.* 1971). Wall regeneration in these cases has been observed in the

light microscope or has been inferred from the characteristics of the regenerating protoplasts. There are few extensive fine structural studies of the wall regenerated by isolated protoplasts (Pojnar *et al.* 1967, Willison and Cocking 1972). Horine and Ruesink (1970) reported the production of an amorphous material around protoplasts isolated from cultured *Convolvulus* tissue. Prat and Roland (1971) showed that mechanically isolated onion bulb protoplasts can produce fibrils.

Protoplasts have been isolated from suspension culture cells of "Pauls's scarlet" rose (Pearce 1972, and in Evans and Cocking 1973). Their survival and regeneration are described below and comparisons are made with the cultured cells from which they were isolated.

2. Materials and Methods

2.1. Culture of Cells

Suspension cells of "Paul's scarlet" rose were grown in a medium (Nash and Davies 1972): KCl, 750 mg/l; $MgSO_4 \cdot 7\,H_2O$, 250 mg/l; $NaNO_3$, 850 mg/l; KH_2PO_4, 140 mg/l; $CaCl_2 \cdot 4\,H_2O$, 110 mg/l; $MnSO_4 \cdot 4\,H_2O$, 1.0 mg/l; $ZnSO_4 \cdot 7\,H_2O$, 0.5 mg/l; H_3BO_3, 0.2 mg/l; Kl, 0.1 mg/l; $CuSO_4 \cdot 5\,H_2O$, 0.02 mg/l; $NaMoO_4 \cdot 2\,H_2O$, 0.02 mg/l; $CoCl_2 \cdot 6\,H_2O$, 0.01 mg/l; ferric citrate, 5.0 mg/l; pyridoxine hydrochloride, 0.5 mg/l; nicotinic acid, 1.0 mg/l; calcium pantothenate, 1.0 mg/l; thiamine hydrochloride, 0.5 mg/l; 2,4—dichlorophenoxyacetic acid, 1.1 mg/l; kinetin, 0.5 mg/; myo-inositol, 100 mg/l; sucrose, 20 g/l. The pH was adjusted to 6.0. Every two weeks an aliquot of the mature culture was diluted 1 : 20 with fresh medium to make 110 ml of suspension. This was cultured in a 250 ml Erlenmeyer flask on a shaker rotating at 90 rpm with a 2 inch horizontal throw at 25 °C.

2.2. Estimation of Fresh Weight, Packed Cell Volume, and Cell Number

Cells were collected in a weighed filtration tube (Davies 1971) and centrifuged at 325 g for 5 minutes. Reweighing gave reproducible fresh weights (*ibid.*). Packed cell volume could be determined using a calibrated filtration tube. There was, however, some variation due to withdrawal from the sides of the tube. Fresh weight data was, therefore, converted to packed cell volume units to obtain an estimate of volume. Fresh weight and packed cell volume was linearly related throughout the growth cycle of the cells and 1 g fresh weight was equivalent to 2.2 ml packed cell volume.

Weighed samples of recently harvested cells, not exceeding 0.5 g per sample, were treated according to the procedure of Henshaw *et al.* (1966) with 20 ml of a chromium trioxide solution to obtain estimates of cell number. More than 600 cells were counted for each sample, with a standard deviation which represented less than 2% of the estimated cell density.

2.3. Isolation of Protoplasts

One gram fresh weight of "Paul's scarlet" rose suspension cells was added to 20 ml of 12% sucrose and 3% partially purified Onozuka 3,000 cellulase (Evans and Cocking 1973) in a 100 ml Erlenmeyer flask and incubated for 24 hours at 33 °C. The released protoplasts were separated from enzyme and debris by centrifuging at 300 g for 1 minute. The protoplasts were transferred from the surface of the centrifuged mixture of enzyme and

protoplasts and were washed by adding to 12% sucrose and recentrifuging. They were similarly washed a second time and transferred to culture medium.
Contaminating cells could be detected in the light microscope and also by inspection of the floating layer of protoplasts obtained after centrifugation.

2.4. Culture of Protoplasts

Protoplasts were cultured in the medium described above with the addition of sucrose as a plasmolyticum and sometimes solidified with 1% Oxoid No. 3 agar. The culture vessels were 100 ml Erlenmeyer flasks, for liquid culture, and petri dishes for culture in solid medium. The culture vessels were not shaken. Culture was at 25 °C in 2.5 foot candles.

2.5. Estimation of Protoplast Density

Aliquots of the protoplast suspension were placed in a counting chamber of known depth (HENSHAW *et al.* 1966). The number of protoplasts in fields of known area were counted, hence the number in a given volume could be calculated. The standard deviation represented less than 9% of the estimated density.

2.6. Light Microscopy

A Zeiss "Photomicroscope" was used for observations with Nomarski interference optics (*cf.*, ROBERTS and NORTHCOTE 1970). To detect cellulose, the protoplasts were treated with 0.5% Calcofluor White (HARRINGTON and ROPER 1968) and illuminated with ultra violet light using a Leitz "Ortholux" microscope. Calcofluor fluoresces with chitin as well as cellulose (*ibid.*).

2.7. Electron Microscopy

Material was fixed for 24 hours in 6% glutaraldehyde and 0.1 M phosphate buffer, pH 7.0, and an equal part of 12% sucrose. The material was washed twice in 0.1 M phosphate buffer, pH 7.0, containing 12% sucrose, and fixed for 1 hour in 0.1% osmium tetroxide in 12% sucrose and 2% calcium chloride. The material was washed three times in 12% sucrose containing 2% calcium chloride. The fixed protoplasts were dehydrated with ethanol, stained in freshly prepared 1% uranyl acetate in ethanol for 15 minutes and then washed with ethanol. Embedding was in methacrylate styrene (MOHR and COCKING 1967). Gold/silver sections were cut with a glass knife on a Porter-Blum MT II ultramicrotome and expanded with trichloroethylene. The sections were post-stained with lead stain (REYNOLDS 1963) (1.33 g lead citrate, 1.76 g sodium citrate, and 8 ml N NaOH in 50 ml distilled water) and inspected in an AEI EM6B microscope at 50 kV with a 50 micron objective aperture.

3. Results

3.1. Isolated Protoplasts in Culture

3.1.1. Survival, Frequency of Division, Long Term Regeneration

Protoplasts were cultured in the medium used to culture the parent cells with the addition of an osmotic stabilizer. Sucrose levels of 12 and 24%, in addition to the 2% already in the medium, were used.
Cultures of protoplasts isolated from actively dividing suspension cells of rose accumulated divided protoplasts during the first four days of culture in

liquid medium plus 12% sucrose (Fig. 1). Similarly cultured protoplasts from non-dividing suspension cells, from a culture which had completed its division phase several days previously, accumulated divided protoplasts over twelve days. This difference can be attributed to the lag expected in the latter case, when protoplasts are transferred from non-division inducing conditions, before isolation, to division inducing conditions, after isolation.

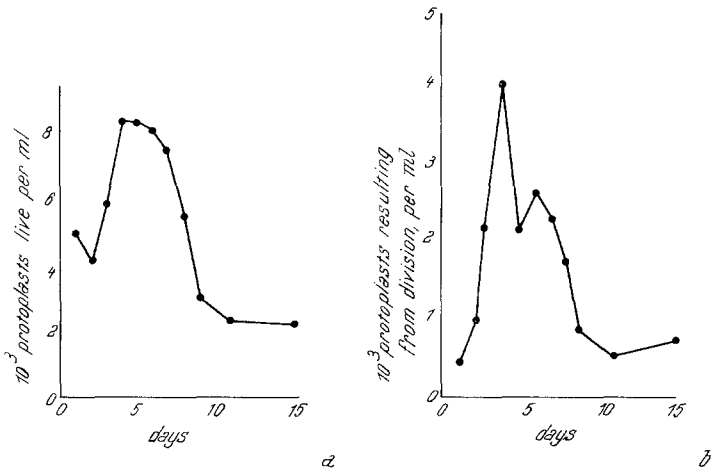


Fig. 1. Protoplasts isolated from actively dividing (log phase) rose suspension culture cells and cultured in medium plus 12% sucrose: change in number of live protoplasts (*a*) and divided protoplasts (*b*) per ml with time

In these liquid cultures, divided protoplasts were less common when the density of protoplasts was above 1×10^5 per ml or below 1×10^3 per ml. In similar medium solidified with agar, dispersed protoplasts at densities as high as 5×10^5 per ml divided at optimal frequency. This difference is due to the locally high density in a layer of protoplasts floating in liquid medium which is avoided in solidified medium.

Reproducibly, 46% of protoplasts cultured at an appropriate density in medium plus 12% sucrose were daughters of protoplast divisions as judged by their occurrence in pairs. The number of protoplasts, however, often almost doubled (Fig. 1), suggesting that some daughters of division separated subsequently. If the level of additional sucrose was increased to 24% the number of protoplasts resulting from division, as judged by their occurrence in pairs, fell to 3% or less and there was often no significant increase in number.

The protoplasts survived slow osmotic pressure reduction over many weeks. Sometimes, cell cultures with growth and wall characteristics similar to those of the parent cells were obtained.

3.1.2. Mode of Division

The two daughter protoplasts resulting from division each contained a nucleus (Fig. 5). Mitosis was followed in a protoplast from prophase to interphase using Nomorski interference optics. Other possible modes of nuclear division, such as constriction into two, were not observed.

It is relevant to ask if mitosis normally preceded cytokinesis in these protoplasts. A possible source of divided protoplasts were the multinucleate protoplasts formed by spontaneous fusion during isolation (PEARCE, in EVANS and COCKING 1973). To test this possibility, protoplasts mostly containing one or more than one nucleus were separately cultured. The mononucleate protoplasts were isolated from cultures of non-dividing cells and the multinucleate protoplasts were isolated from cultures of dividing cells (*ibid.*). Divided protoplasts occurred almost as frequently in cultures containing nearly all mononucleate protoplasts as in cultures containing a high proportion of multinucleate protoplasts (Table 1). The number of multinucleate protoplasts did not significantly diminish during culture.

Table 1. *Occurrence of Divided Protoplasts in Cultures of Largely Mononucleate and Multinucleate Protoplasts*

% protoplasts containing more than one nucleus when isolated	% protoplasts resulting from division during culture
86	47
39	44
2	36

During cytokinesis, the phragmoplast was initially present but was absent later (Fig. 2). The cell-plate initially appeared normal in the light microscope but later appeared as a deep tongue into the vacuole (Fig. 2). The cytoplasm at the tip of this tongue fused with the cytoplasm lining the cell (Fig. 3). In the electron microscope, the developing cross-wall appeared as a fibril-lined (Fig. 7) or fibril-filled tongue. This abnormal cytokinesis occurred even after several weeks in culture in medium plus 12% sucrose, by which time some wall encircled each protoplast.

Cytoplasmic strands were absent during cytokinesis (Fig. 2) and division was often into two very unequal parts (Fig. 8).

3.1.3. Wall Regeneration

During the first few days in culture no wall material was revealed around the protoplasts either by calcofluor treatment or in the electron microscope.

It is possible that during this period very small amounts of material were produced but these were not detected by the methods used (*cf.*, Cocking 1972). By ten days of culture in rose medium plus agar and 12% sucrose, significant quantities of fibrillar material and some non-fibrillar material occurred around most protoplasts (Fig. 6). The regenerated wall fluoresced blue when treated with calcofluor (Fig. 4).

Non-fibrillar material was present (Figs. 6 and 7) but was not the predominant component of wall produced by these protoplasts. A wall containing a higher proportion of non-fibrillar material was formed by protoplasts in liquid medium plus 12% sucrose. Protoplasts cultured in liquid medium plus 24% sucrose took four weeks to regenerate a wall which fluoresced with calcofluor but an envelope was detectable in the light microscope before then. In this medium fibrillar material was produced with very little other material by some protoplasts but most developed a wall composed mainly of non-fibrillar material (Fig. 10).

3.1.4. Relationship between Wall Regeneration and Cytokinesis

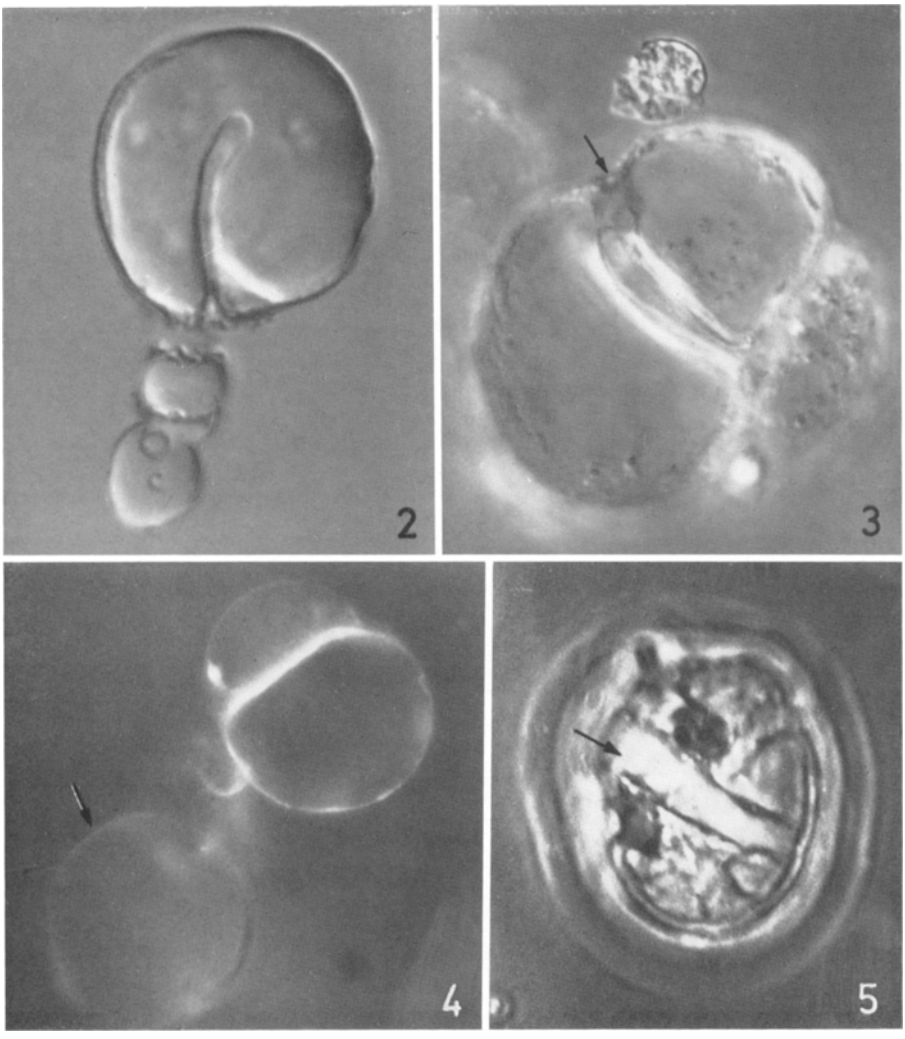
Protoplasts were cultured in medium plus 24% sucrose and solidified with agar. They were treated after four weeks with calcofluor. Fluorescence was rare around undivided protoplasts. On the other hand, the cross-wall and periphery of divided protoplasts was always fluorescent. Clearly, there was a connection between protoplast division and the formation of a peripheral wall component.

Cytokinesis was followed in a three day old protoplast cultured in liquid medium plus 12% sucrose. The periphery did not initially fluoresce with calcofluor. The new cell plate fluoresced and the fluorescence spread to a groove-like shape (Fig. 5).

3.1.5. Expansion

Protoplasts cultured in liquid medium plus 12% sucrose were initially spherical but after a few days became elongate (Fig. 8). After a week many budded (Fig. 9). When protoplasts were cultured in rose medium plus 24% sucrose budding was common only after three weeks. In medium solidified with agar budding was not frequent. The number of nuclei per budded protoplast was one when originally mono-nucleate protoplasts were cultured.

Protoplasts were cultured in rose medium plus 12% sucrose. The longest and shortest axes of twenty undivided protoplasts were measured after three and five days culture (Table 2) before budding made measurement impossible. If the tendency to elongate is ignored, and it is assumed that these protoplasts expanded as perfect spheres, then it can be calculated that they increased their volume from day 3 to day 5 at the rate of 100% per day over their day 3 size. This corresponds to an increase in surface area of 2,232 μm^2.



Figs. 2–5. Light micrographs of protoplasts cultured in medium plus 12% sucrose. Fig. 2. Cytokinesis. Note absence of phragmoplast and cytoplasmic strands. Nomarski interference optics. ×1,100; Fig. 3. Late cytokinesis. Shows fusion of cytoplasm at tip of growing tongue-like cross-wall with cytoplasm around the vacuole (arrow). Note width of cross-wall. Nomarski interference optics. ×1,100; Fig. 4. Protoplast cultured for ten days in medium solidified with agar. Treated with calcofluor and illuminated with ultra violet light only. Note bright fluorescence of cross-wall and fainter fluorescence of undivided protoplast (arrow). ×490; Fig. 5. Protoplast cultured for three days, treated with calcofluor and illuminated with ultra violet light, and visible light using phase condenser and objective. Note nuclei to either side of a fluorescent band which is the new cross-wall (arrow). ×670

Twenty protoplasts cultured in medium plus 24% sucrose were also measured on each of several days (Table 2). The dimensions recorded increased more slowly than those of the protoplasts cultured in the medium containing less sucrose.

Table 2. *Average Dimensions of Twenty Protoplasts Sampled from Cultures of Different Ages*

	Medium plus 12% sucrose		Medium plus 24% sucrose	
Age of culture (days)	Average length of long & short axes (μm)	Difference between long & short axes (μm)	Average length of long & short axes (μm)	Difference between long & short axes (μm)
3	37	1.2	33	0
5	53	6.6	31	1.6
7	58	10.9	39	3.6
12			41	1.7

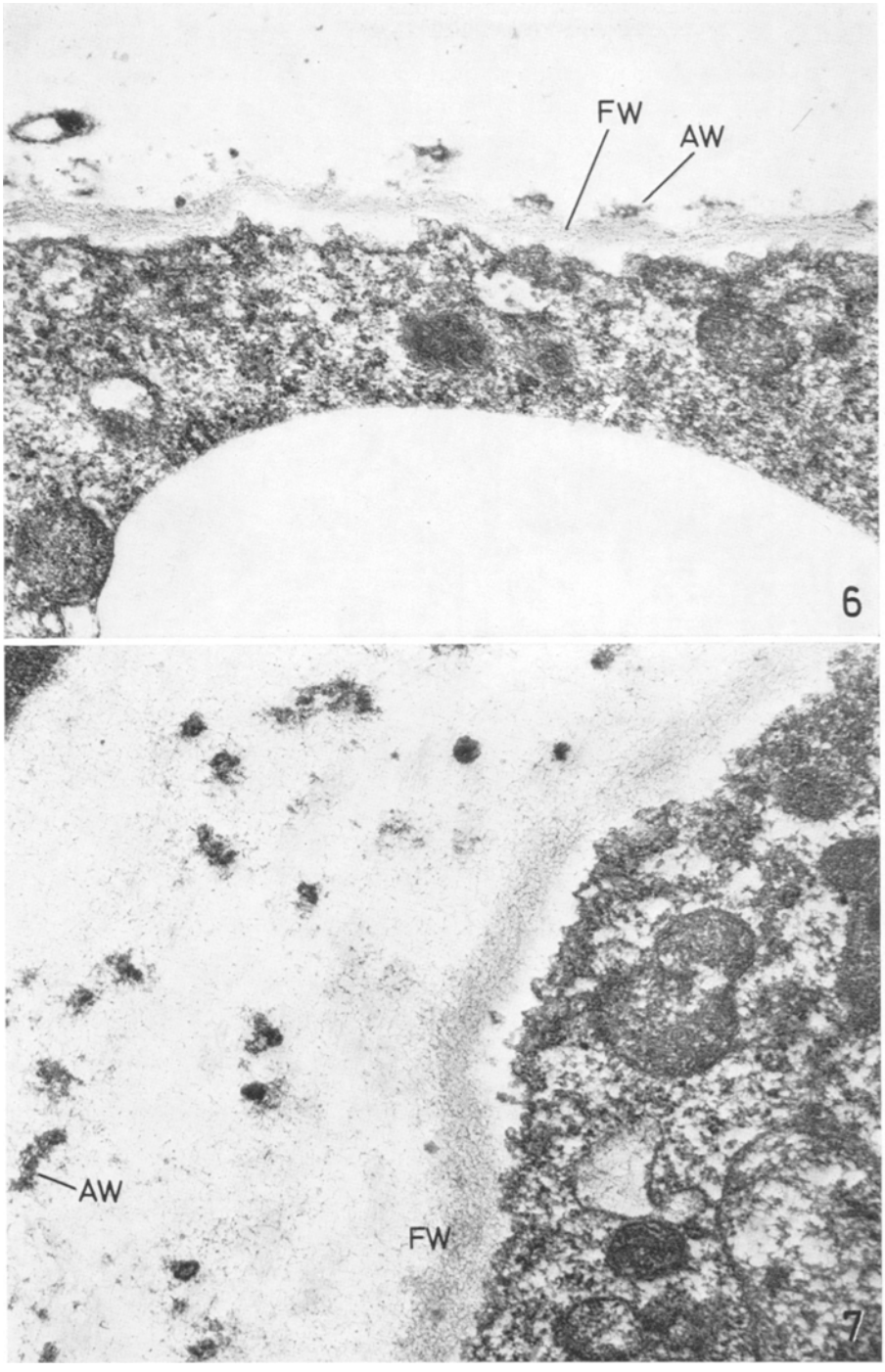
3.2. Cells in Culture

3.2.1. Cultures of Cells in Non-Plasmolysing Medium

Cells were cultured in 100 ml of medium, without further additions, in Erlenmeyer flasks on the rotary shaker. Packed cell volume and cell number were estimated. There was a doubling in cell number every 1.3 days during the phase of division. The results indicated an increase in volume per cell in the period immediately after the end of the division phase of not more than 22% per day of the previous cell volume. During the division phase when the rate of cell number increase was maximal (one doubling in 20 hours), the average increase in volume per cell between divisions was calculated as 57% per day of the previous volume.

The division phase cells were assumed to expand as perfect spheres and the stationary phase cells were assumed to be perfect cylinders expanding only in the direction of the cylindrical axis. The division phase cells increased their surface areas, on the basis of cell number and packed cell volume data, by 2,200 μm^2. This is an overestimate since packed cell volume is a measure

Figs. 6 and 7. Electron micrographs of parts of isolated protoplasts cultured for ten days in medium plus 12% sucrose solidified with agar. Fig. 6. Note regenerated wall composed of fibrils (*FW*) and, to the outside, amorphous material (*AW*). ×30,000. Fig. 7. Region of cross-wall. Note presence of fibrils (*FW*) and amorphous material (*AW*) in the wall. ×30,000



Figs. 6 and 7

of cell volume plus space between the packed cells. On the basis of measurements made of twenty cells from a day 17 culture and the percentage increase in surface area calculated from packed cell volume data an actual increase in surface area of 1,400 μm^2 per day was calculated for stationary phase cells. During cytokinesis the phragmoplast crossing the vacuole was connected by cytoplasmic strands to other parts of the cell (Fig. 11) and divided the cell into two approximately equal parts.

Table 3. *Rose Cells Grown in Plasmolysing Medium (Medium plus 12.6% Sucrose): Changes in Fresh Weight per 100 ml Culture with Time*

Age of culture (days)	Fresh weight (g)
6	0.52
11	0.71
14	1.23
18	1.45
22	1.67
27	2.38
33	3.48
41	3.89
53	4.39
81	5.15

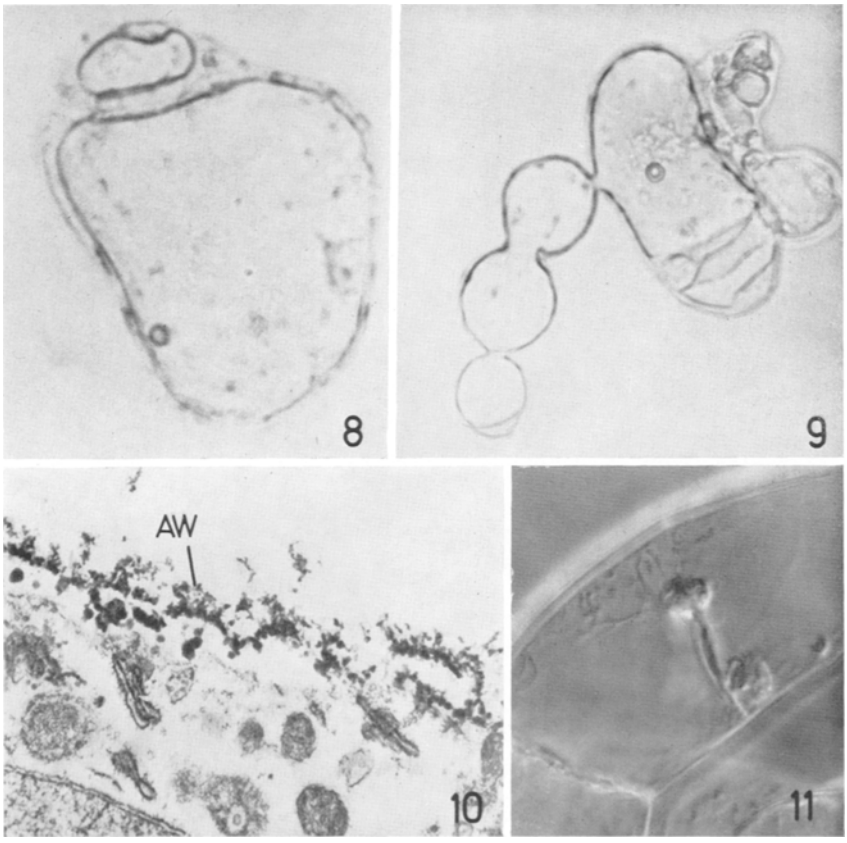
3.2.2. Cultures of Cells in Plasmolysing Media

Cells were inoculated into 100 ml of medium plus 12.6% sucrose and cultured on the rotary shaker in Erlenmeyer flasks. Some divisions were observed. It was calculated from the data obtained (Table 3) that between days 11 and 33 the cells increased in volume by 18% per day, of the day 11 volume, assuming no division to have occurred. The fresh weight of cultures of cells in medium plus 24% sucrose increased from 0.7 g to 1.5 g per culture in 90 days.

4. Discussion

Extensive studies (PEARCE 1972, and in EVANS and COCKING 1973) have been made of factors affecting release and survival of rose protoplasts. The pragmatic approach to protoplast culture, that they should grow in a medium in which the parent cells will grow, needs to be studied in terms of these

factors. Additions of RNA, protein and lipid to the enzyme incubation to protect the protoplasts partly against possible enzymic impurities (COCKING 1972) did not improve survival (PEARCE 1972). Partial purification of the enzyme, which removes salts and phenols, did not significantly affect the



Figs. 8–11. Fig. 8. Light micrograph of isolated protoplast cultured in medium plus 12% sucrose. Note very unequal division, and elongation of protoplast. Bright field. ×650; Fig. 9. Light micrograph of isolated protoplast cultured in medium plus 12% sucrose. Note budding and continuity of vacuoles between buds. Bright field. ×290; Fig. 10. Electron micrograph of part of an isolated protoplast cultured for forty days in medium plus 24% sucrose. Note quantities of amorphous wall material (*AW*). ×19,000; Fig. 11. Light micrograph of cytokinesis in cell cultured in non-plasmolysing medium. Shows phragmoplast and developing cross-wall. Note cytoplasmic strands between phragmoplast and cytoplasm lining the vacuole. Nomarski interference optics. ×510

incident of divisions (*ibid.*). The reason is not clear why wall degrading enzymes or their impurities should affect cytokinesis in some protoplasts (ERIKSSON and JONASSON 1969) but not in others (TAKEBE *et al.* 1971).

The fluorescence of the regenerated wall with calcofluor and the appearance

of fibrils in the electron microscope suggests that cellulose is an important component of the regenerated wall, although chitin remains a possibility. Regenerated walls also contain a non-fibrillar component whose nature is not clear but which might include some cytoplasmic debris. Increased sucrose level reduces the accumulation of fibrillar and calcofluor fluorescent material, perhaps due to reduced rate of wall polysaccharide synthesis (Ordin 1960, Greenway and Leahy 1970).

The wall regenerated by rose protoplasts differs from the wall produced by tomato fruit locule tissue protoplasts, which often has a substantial multilamellar system as well as fibrillar material (Pojnar *et al.* 1967, Willison and Cocking 1972). It also differs, except when rose protoplasts are cultured with 24% sucrose in liquid medium, from the wall of amorphous material which Horine and Ruesink (1970) reported that isolated convolvulus callus protoplasts produce. Some fibrils, however, can generally be found amongst the non-fibrillar material in rose, and some protoplasts in medium plus 24% sucrose produce mainly fibrils. It is clear that isolated leaf protoplasts can produce a largely fibrillar wall (Cocking and Grout, personal communication). It appears (Eriksson and Jonasson 1969, Hellmann and Reinert 1971) that, while chloroplast containing protoplasts are usually successful in rapidly establishing a wall, protoplasts from tissue culture material may be slower, and some form a wall apparently without cellulose (*cf.*, Horine and Ruesink 1970) or no wall at all (Hellmann and Reinert 1971). It is not clear why this is but it might result from the physiology or structure of the parent cell. It is possible, for instance that plasmolysis during isolation separates some protoplasts from important enzymes located in the wall (*cf.*, Prat and Roland 1971).

The abnormality of cytokinesis in rose and its absence despite the occurrence of mitosis in protoplasts from some tissue culture materials (Eriksson and Jonasson 1969, Hellmann and Reinert 1971, Bawa and Torrey 1971) suggests that cytokinesis is also affected by the factors which affect wall formation. In fungal protoplasts, there is no division until a wall is formed (Nečas 1971). A similar phenomenon might occur in higher plant protoplasts (Cocking 1972). In the case of rose protoplasts, the circumstantial evidence, early division and slow wall formation, suggests that the prior existence of a peripheral wall is not required for cytokinesis. On the contrary in medium plus 12% sucrose or 24% sucrose cytokinesis probably hastens formation of that wall component which fluoresces with calcofluor. This corresponds to the general case (Kreger 1969).

These various results nevertheless suggest that there is a dependence of cytokinesis on the presence of wall or wall producing capacity. Normally in rose cells, cytoplasmic strands link the phragmoplast across the vacuole to other parts of the cell (Fig. 11). In the protoplast these are absent (Fig. 2). It may be that essential support for the phragmoplast is thus lost and this

may be why it disappears. This may explain the frequent unequal division of the protoplasts (Fig. 8), even though the cells are normally divided into two equal parts (NASH and DAVIES 1972). It would follow from this that the orientation of the plane of mitosis could not solely determine the orientation of the plane of cytokinesis. BERGMANN (1960) observed that, in the absence of strands the cross-wall did not form at right angles to the long axis of the cell. DAS *et al.* (1966) found that in cultured tobacco cells cytoplasmic strands determined the direction of growth of the cross-wall during cytokinesis and concluded that in their absence cytokinesis would not occur. Strands are also seen supporting nuclei and surrounding cytoplasm during mitosis (*ibid.*, ROBERTS and NORTHCOTE 1970) and may be of structural importance then. An effect of stress on the orientation of division in cultured artichoke tissue has been observed by YEOMAN and BROWN (1971) and they attribute this to the reception of stress in the plasmalemma due to some association between plasmalemma and wall, in which the stress primarily occurs. They also found a similar effect due to plasmolysis. Thus cross-vacuolar strands have been implicated in cytokinesis, and the relationship between wall and plasmalemma may have some importance too. The results with rose protoplasts suggest a causal connection between the relationship of cytoplasm to wall and the presence, and hence perhaps also the distribution, of cross-vacuolar strands controling the plane of division. The nature of the relationship between wall and cytoplasm would in that case be of great interest. The association of endoplasmic reticulum with plasmadesmata might provide sufficient connection between the wall and cytoplasm. If the abnormal cytokinesis in rose is due to initial formation of a cell plate and subsequent failure to extend this in a normal way due to the disappearance of the phragmoplast, then it follows that the cross-wall tongue is extended by some other means than the coalescence of Golgi-derived vesicles. These means may be a plasmalemma growth paralleled by cellulose deposition and which could be similar to plasmalemma growth during normal cell expansion. This also implies that in those cases in which protoplasts do make some wall but mitosis occurs without cytokinesis (ERIKSSON and JONASSON 1969, BAWA and TORREY 1971), there is probably failure in the initial formation of the cell plate. This might be due to an effect of osmotic pressure on Golgi activity but it is also possible that the factor which leads to the disappearance of the phragmoplast in rose affects cell plate formation at an earlier stage in these other protoplasts.

There have been no previous comparisons of the growth rates of isolated protoplasts and cells of the same origin in similar media. Reports of the effect of high osmotic pressures on the growth of roots (BURSTRÖM 1953, GONZÁLEZ-BENÁLDEZ *et al.* 1968) and embryoids (AMMIRATO and STEWARD 1971) indicate that division need not be greatly affected but expansion may be much reduced. The division rate of the protoplasts in medium plus

12% sucrose is at least half that of the parent cells in non-plasmolysing medium and is greatly reduced when the additional sucrose is increased to 24%. The rate of expansion, measured as percentage volume increase, however, is greater in protoplasts than in plasmolysed or non-plasmolysed cells. No doubt this result is due to absence of wall around the protoplast.

The difference in growth of rose protoplasts in medium plus 12% and 24% sucrose, which can be reproduced with similar molar amounts of mannitol (Pearce 1972), shows that removal of the wall is not the sole factor involved in protoplast development: osmotic pressure affects expansion, division and wall regeneration of isolated rose protoplasts. If the results with rose are of general applicability then it can be said that to achieve adequate division rates it may be necessary to use the lowest osmotic pressure compatible with the protoplasts survival. Moreover since the higher osmotic pressure reduces accumulation of fibrillar and calcofluor fluorescent material and appears also to affect the composition of the wall, using the lowest osmotic pressure may also result in better wall formation.

Rose protoplasts, although having little wall during the first weeks of their culture in liquid medium, do not expand unhindered. Restriction occurs with the development of the wall. Weak areas may be responsible for the budding which is seen in cultured rose protoplasts (Fig. 9) and other protoplasts (Bawa and Torrey 1971, Motoyashi 1972). This budding is an indication that, while sufficient wall is present to affect the position of expansion, the extent of that expansion is not limited by the wall. The concept of Nečas (1971) for yeast protoplasts that "The morphology of reversion to normal forms seems to depend on the proper balance between the rate of wall regeneration, and the rate of growth of the protoplast", is thus also applicable to higher plant protoplasts. Since wall is also implicated in normal cytokinesis the capacity for substantial wall formation can be considered as of primary importance in higher plant protoplast culture.

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Authors' address: Dr. R. S. PEARCE, Department of Plant Physiology, Biology Building, University of Sussex, Falmer, Brighton, BN 1 9QG, U.K.