

Cell Wall Regeneration around Protoplasts Isolated from *Convolvulus* Tissue Culture¹

Received for publication September 15, 1971

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ABSTRACT

Protoplasts of *Convolvulus arvensis* L. tissue culture regenerated a wall-like structure within 3 days in culture. Although unusually electron dense and atypically amorphous in the electron microscope, this structure could be digested with *Myrothecium* cellulase but was resistant to protease, a Rohm and Haas pectinase, and a β -1,3-exoglucanase just like the original wall. A cytochemical test for callose was negative. Wall regeneration required a readily metabolized external carbon source and was not inhibited by a high concentration of cycloheximide, puromycin, or actinomycin D. Protoplast budding was correlated with the wall regeneration, and the latter was related quantitatively to the sucrose concentration in the medium. Although a concentration of 1 μ M 2,4-dichlorophenoxy acetic acid is used normally for both general culture of the tissue and for wall regeneration, concentrations of 0 and 0.1 mM, which are highly deleterious to growth, have no appreciable effect on the incidence of the wall-like structure regenerated around protoplasts. The ability of protoplasts to undergo cell wall regeneration was decreased when they were cultured in the presence of proteolytic enzymes.

The regeneration of a wall-like structure around protoplasts devoid of cell wall has been observed for yeast (12), other fungi (2, 15), mosses (6), algae (16, 40, 43), tomato fruit (22, 29), *Haplopappus* suspension culture (13), tobacco mesophyll cells (24), soybean cell suspensions (20), onion root cells (33), maize endosperm cells (23), and *Convolvulus* cells (4, 19). Precise laboratory control of such wall regeneration would facilitate both studies of wall synthesis itself and the use of protoplasts for somatic hybridization experiments (32). In protoplast fusion work, it is useful to maintain a totally wall-less protoplast surface until desired fusions are accomplished, then to initiate rapid wall synthesis. Few methods are yet known for such control (10). No previous work has sought the relationships between the culture conditions and the occurrence of the resulting WLS.³ The question of the contribution of pre-existing wall components to the control of the biosynthesis of new wall has

not been answered, and this work provides a unique approach to the question.

In the present work, the phenomenon of bud formation by *Convolvulus* protoplasts has been correlated more carefully than ever before with the regeneration of a WLS, indicating to what degree the budding noted here and in previous papers (4, 8, 20, 23, 24) is indicative of wall regeneration at the plasma membrane. Since it has been impossible to amass enough WLS for a straightforward biochemical analysis, a partial characterization of the WLS has been performed by selective enzymatic digestion.

MATERIALS AND METHODS

Protoplasts from *Convolvulus arvensis* tissue culture (11) were prepared by treating tissue for 2 hr with 4% cellulase obtained from *Myrothecium verrucaria* (35, 36) in an osmoticum of 0.14 molal KCl and 0.10 molal CaCl₂. Molal solutions rather than molar were used throughout for the osmotic solutions, since osmotic potential is proportional to molal concentration. The difference between molal and molar strength is insignificant for the ionic solutions but is about 5% for 0.5 molar and molal mannitol solutions and 10% for 0.5 molar and molal sucrose solutions. Cells were taken from a culture on agar medium inoculated 2 to 3 weeks previously. Sterilization of the enzyme solution was accomplished by micro-syringe Millipore filtration using Solvint filters. The protoplast preparation was washed twice with 10 volumes of 0.14 molal KCl + 0.10 molal CaCl₂ with centrifugation between washings for 1 min at 50g. Subsequently, protoplasts were cultured in sterile plastic 5-cm Petri dishes (Lab Tek No. 4036) containing 5 ml of a standard medium in 0.7% agar, the "underlayer." The standard medium composition was that used by Earle and Torrey (11) and contained salts, yeast extract, and 1.0 μ M 2,4-D at pH 5.5, with the sucrose concentration raised to 0.5 molal. No cytokinin was required.

A 1-ml "overlayer" of salts (0.10 molal CaCl₂ + 0.14 molal KCl) without agar or nutrients was pipetted onto the solidified underlayer. Protoplasts in 50 μ l of solution were pipetted directly into the overlayer, which was about 0.5 mm thick and prevented their drying out. For culturing, dishes were covered with a black cloth and placed in a damp chamber having an atmosphere saturated from a solution of the same ionic composition as the overlayer.

After being cultured for 5 to 6 days, some protoplast cultures were scored for budding by a systematic scanning of the culture dish under a bright-field compound microscope at 100 \times . Such data are reported as the percentage of the total number of protoplasts counted that showed distinct budding. In other cases, the presence of a WLS was determined directly by adding 2 ml of 2 molal KCl directly to the overlayer and scoring at

¹ The National Science Foundation provided support through Grant GB 8006. This material was included in a doctoral thesis submitted by R. K. Horine to the Graduate School of Indiana University.

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³ Abbreviation: WLS: wall-like structure.

100× any cells with wall structure left after the plasmolysis of the cytoplasm. All the values reported in the tables or text represent an average of at least two dishes in which each dish contained between 100 and 600 protoplasts.

Only those protoplasts that were isolated free of any other protoplasts were counted. Under a light microscope, single free protoplasts are always seen to be completely devoid of their native cell walls. On the other hand, clumps of three or more protoplasts often have some remaining wall material around them, so these were never included in the results.

The proteolytic activity of trypsin and pronase solutions was measured both immediately after being prepared and after being exposed to protoplasts for 6 days. The substrate used for the assay was 2.0 ml of 2.5 mg/ml bovine serum albumin buffered with 20 mM phosphate-citrate at pH 6.8. To start the assay, 0.01 ml of pronase or trypsin was added to the buffered bovine serum albumin solution and incubated for 30 min at 25 C. After this time, trichloroacetic acid was added to 4%, and the solution was placed on ice for 5 min before the precipitate was spun down. The amount of protein in the supernatant was determined by the Lowry method (21) and was used to compare the relative activities of the various proteolytic enzyme solutions.

Photographs of the protoplasts were taken with a Zeiss Standard Universal microscope under bright field illumination using a green filter with 3-1/4" by 4" sheets of Kodak Contrast Process Panchromatic film in a Leitz Aristophot camera.

Tissue or protoplasts for electron microscopy were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2 for 2 hr at 3 C. After being embedded in agar, cells were postfixated in osmium, dehydrated with ethanol and propylene oxide, and embedded in Epon. Sections were cut with glass knives, poststained in uranyl acetate and lead citrate, and examined in a Hitachi HU-11C electron microscope.

Sources of Enzymes and Reagents. Actinomycin D (Mann Research Laboratories), bovine serum albumin, A grade (Calbiochem), cycloheximide (Sigma Chemical Company), 2,4-D (J. T. Baker Chemical Company), pronase, B grade (Calbiochem), puromycin (Nutritional Biochemicals Company), trypsin-pancreatic, A grade (Calbiochem), kinetin (Calbiochem), Glucostat Kit (Worthington Biochemicals), Rhozyme HP-150 "Pectinase" (Rohm and Haas). This Rhozyme was supplied as a "cellulase," but was used as a pectinase, since it contained less than 3% of the activity on swollen cellulose of the *Myrothecium* cellulase preparation and over five times the activity of another pectinase preparation in reducing the viscosity of polypectate. The β -1,3-exoglucanase was a gift from D. E. Eveleigh, Rutgers University.

RESULTS

Initially, the observation that some protoplasts budded and others did not suggested that counting the protoplasts budding might be an easily quantitated method for detecting the number of protoplasts regenerating a wall under different culture conditions, so long as it could be shown that most protoplasts regenerating a WLS in fact budded and those not regenerating a WLS did not. As will be shown, this correlation proved not to hold for situations where the wall becomes sufficiently strong to prevent the budding; therefore, it became necessary to develop a more direct method of detecting which protoplasts were regenerating a WLS. By osmotically shrinking the protoplasm and using appropriate microscopic conditions, it proved possible to see the WLS around protoplasts and to count directly the fraction of protoplasts exhibiting WLS formation (see Fig. 6).

WLS Formation, Protoplast Budding, and the Control of These Two Processes. *Convolvulus* protoplasts exhibit budding after being cultured for 2 to 3 days under the standard conditions, with sucrose, dilute salts, 2,4-D, and agar in the underlayer and the ionic osmoticum in the overlayer. As shown in Figures 6 and 7A, bud formation refers not to cellular division but rather to a protrusion of the protoplast surface. In order to determine the correlation between budding and WLS formation, cultures were scored both for budding and for the presence of a WLS after plasmolysis. Scoring for budding was easier than scoring for WLS regeneration. Initial stages of WLS formation in unbudded protoplasts were difficult to detect. In some cases, the wall collapsed and remained attached to the plasma membrane during plasmolysis, and in other cases the WLS was so thin that detecting it at low magnification with the light microscope was difficult. Table I shows the absence of any budding activity when protoplasts were cultured under a condition that would not stimulate the production of a WLS. This condition was obtained by omitting sucrose from the underlayer and replacing it by an osmotically equivalent concentration of ionic osmoticum. The possibility that the ionic environment during this time caused some irreversible disruption of the cell's ability for regeneration of a WLS was eliminated by the second experiment reported in Table I. These data show that when protoplasts were cultured under ionic conditions for 4 to 6 days and then transferred onto an agar underlayer containing the standard medium, the protoplasts still could produce a WLS. In fact, usually a greater fraction than normal of such protoplasts produced a WLS, as manifested by the percentage exhibiting budding. Either sucrose or mannitol in the underlayer was found to stimulate budding and WLS formation. When mannitol was substituted for sucrose in liquid shake cultures of *Convolvulus* callus tissue, growth occurred at about one-half the sucrose-supported rate, indicating that mannitol can be metabolized by the cells.

WLS formation is altered by the concentration of sucrose in the agar underlayer (Fig. 1). The protoplasts were cultured on agar underlayers which possessed differing concentrations of sucrose but which still had an osmolality equivalent to 0.5 molal sucrose, obtained by adding to the underlayer the appropriate amounts of the ionic osmoticum. In the sucrose concentrations shown, the fraction of protoplasts undergoing wall regeneration increased with increasing sucrose concentration. At even higher concentrations of 0.75 and 0.9 molal sucrose, survival and WLS formation were both markedly decreased. Figure 2 presents the results of two experiments comparing the budding activity and the formation of a WLS for protoplasts placed onto underlayers possessing varying amounts

Table I. Budding of Protoplasts Cultured under Ionic and Standard Conditions

To transfer the protoplasts from ionic conditions to standard conditions, the ionic overlayer containing the protoplasts was poured off the ionic agar underlayer onto a standard agar underlayer containing sucrose as the osmoticum.

Days on Ionic Medium	Subsequent Days on Standard Medium	Percentage of Protoplasts Budding
Experiment 1		
0	5	25
5	0	0.2
Experiment 2		
4	6	65
6	7	79

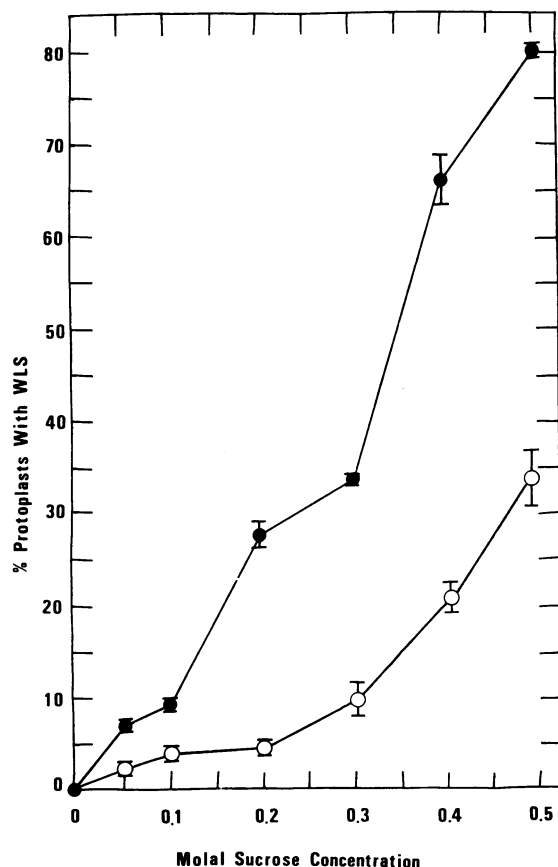


FIG. 1. Effect of different concentrations of sucrose in the agar underlayer on the percentage of protoplasts undergoing the regeneration of a WLS. Protoplasts were cultured for 7 days. Each point is the average of at least four dishes, and the bars represent average deviations. Each curve represents a series with a different batch of protoplasts having different budding potential.

of sucrose. When regeneration was regulated in this way, high budding activity corresponded to a high proportion of protoplasts regenerating a WLS.

The essential component of the agar underlayer for WLS formation was the carbon source. When sucrose was present alone in the agar underlayer, (*i.e.*, no salts, yeast extract, or hormone), the fraction of protoplasts undergoing WLS formation was the same as that for protoplasts cultured over an agar underlayer possessing the complete standard medium. Further proof of the need for a carbon source which can be readily metabolized was obtained by culturing protoplasts over an agar underlayer containing either sucrose which is readily metabolized or glycylglycine which is not metabolized (Table II). When glycylglycine was included in the agar underlayer at a concentration of 0.1 molal with 0.4 molal sucrose, there was no major disruption of regeneration, with the decrease in WLS formation representing that expected by the decrease in sucrose concentration (Fig. 1). The glycylglycine results also indicated that it is the presence of sucrose and not the absence of excess ions that enhances WLS formation. When Carbowax 1500 in an amount osmotically equivalent to 0.5 molal was added to the agar underlayer in place of sucrose, protoplasts did not survive culture.

Protoplasts which have been cultured for a period of 4 to 6 days with the ionic osmoticum in both overlayer and underlayer are often one and one-half to two times larger in diameter than protoplasts cultured for the same length of time with su-

crose present in the agar underlayer. Moreover, the number of protoplasts surviving under ionic conditions after 6 days of culture can be as much as three to six times greater than the number present after 6 days of standard culture.

Ionic Requirement for WLS Formation. The calcium salt of the overlayer was not specifically required. Substitution for the calcium and potassium ionic overlayer of 0.30 molal KCl

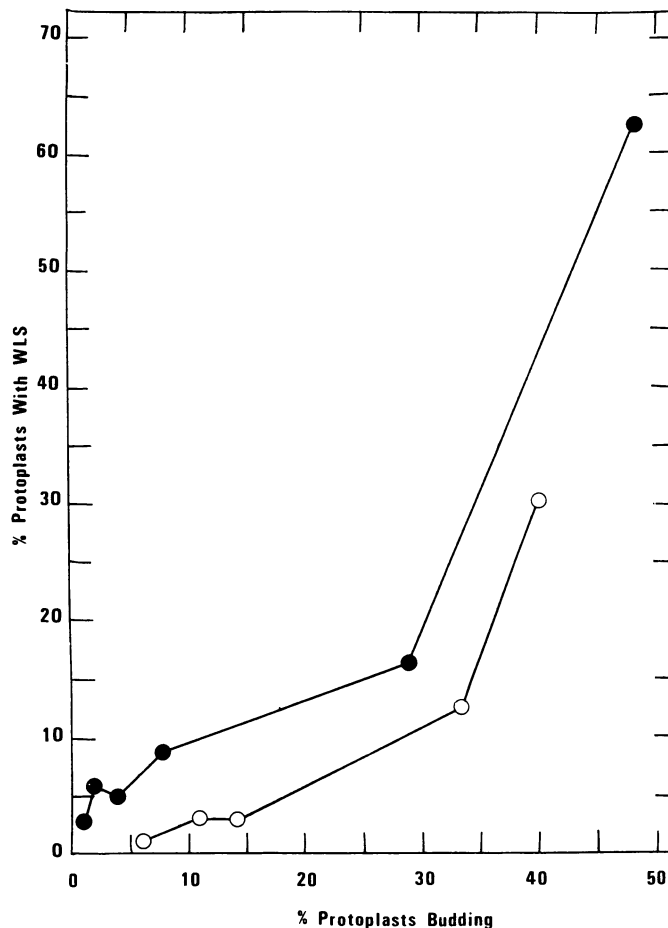


FIG. 2. Relationship of budding to WLS regeneration. Separate experiments in which protoplasts were cultured for either 7 days (○) or 8 days (●) in an ionic overlayer over agar medium containing varying sucrose concentrations. Each point represents the average score for two dishes.

Table II. Requirement for a Utilizable Carbon Source in the Medium for WLS Regeneration

For all experiments, the medium was adjusted to pH 5.7 before autoclaving. Osmotica shown were used alone without other nutrients in the agar underlayer. Protoplasts were cultured for 4 to 7 days before observation.

Medium in the Underlayer	Protoplasts with WLS
	%
Experiment 1	
0.5 molal sucrose	40
0.5 molal glycylglycine	1
Experiment 2	
0.4 molal sucrose + 0.1 molal glycylglycine	17
0.5 molal sucrose	20

alone or 0.10 molal MgCl₂ in 0.14 molal KCl gave no difference in WLS formation for these cells when they were placed on a sucrose underlayer after 6 days in the ionic osmoticum noted. These protoplasts were prepared and washed with the same salt concentration as the overlayer used during culture. Protoplast survival was not hindered by the reduction of calcium concentration to the 1 mM level of the standard underlayer nutrient medium during the 6 days of ionic culture. Neither was the ability to undergo WLS regeneration upon transfer to standard conditions affected by this reduced calcium concentration.

Electron Microscopy of the WLS. Electron micrographs of the intact *Convolvulus* tissue and the protoplasts isolated from it indicate that those portions of the wall that are electron opaque under the present fixation and staining procedures have been completely removed (Figs. 3 and 4). Being embedded in agar, freshly isolated protoplasts do have some loosely fibrillar material in their surrounding medium; however, they consistently show no trace of any wall such as that of Figure 3. Protoplasts incubated 6 days in an ionic osmoticum show no trace of regenerated wall; however, protoplasts cultured on an underlayer containing sucrose produced the WLS shown in Figure 5, an amorphous, highly electron dense material, found both on the surface and sometimes within small vacuoles (note in upper left) resulting from excess membrane material that was once at the protoplast surface (35). The electron micrographs show no obvious origin for the knobby appearance of the WLS seen with the light microscope in most cases when the cytoplasm is plasmolyzed away (Fig. 6).

Effect of Light on WLS Formation. Preliminary observations indicated that light from a south window was strongly inhibitory to the regeneration of a WLS. Thereafter, protoplasts were routinely cultured in the dark, although two experiments in which protoplasts were cultured in light of 340 ft-c from a 60-W incandescent bulb indicated no strong effect of light upon WLS formation, which occurred upon 43% of the illuminated protoplasts compared with 52% of the protoplasts in darkness.

Involvement of RNA Synthesis and Protein Synthesis in WLS Formation. In a preliminary experiment, protoplasts were incubated for 6 days in either puromycin or cycloheximide concentrations up to 90 μ M or actinomycin D concentrations up to 10 μ M in the overlayer. The incidence of budding was monitored and found to be near 30% for all treatments, both experimental and controls. In a second experiment, a pretreatment was given in which protoplasts were incubated 6 days in each of the above inhibitors at 100 μ M with the ionic osmoticum present to prevent WLS formation. Protoplasts were then poured onto an underlayer containing the appropriate inhibitor with sucrose as the osmoticum. In each of the treatments and in the controls, 53 to 59% of the protoplasts exhibited WLS formation when plasmolyzed after 6 more days of incubation. In a 4-hr experiment with the intact tissue, actinomycin D at 10 μ M reduced incorporation of ¹⁴C-leucine into trichloroacetic acid-insoluble material very little; puromycin and cycloheximide at 100 μ M reduced incorporation by 84% and 78% respectively, although uptake was reduced to a significant though lesser extent (Ruesink and Jacobitz, unpublished).

Lack of Residual Wall on the Protoplasts. Protoplasts isolated after a 2-hr treatment with cellulase have never shown any residual wall upon plasmolysis. In contrast, intact cells always show a wall upon plasmolysis, and a regenerated WLS appears as in Figure 6. Under the electron microscope, membranes of freshly isolated protoplasts are free of any recognizable wall material (Fig. 4). On the other hand, one report has indicated the presence of wall residues around "protoplasts"

released by an enzyme treatment of minimum duration (13). To test whether there might be any such residual wall material on these protoplasts acting as a primer for the regeneration of a WLS, protoplasts obtained after longer periods of digestion in cellulase were cultured. Protoplasts were prepared by 2-, 4-, 8-, and 16-hr digestion with *Myrothecium* cellulase and then cultured for 6 days under standard conditions. In no case was there a significant change in regeneration frequency, with results ranging from 68 to 78%. Combined with microscopic evidence for the lack of detectable amounts of wall residues, this evidence indicates that WLS formation takes place in the absence of appreciable amounts of primer left from the previous wall. These data also emphasize the general lack of toxicity of the *Myrothecium* enzyme preparation used to release the protoplasts.

Effects of Proteolytic Enzymes. To assess the participation in WLS formation of enzymes exposed on the plasma membrane surface, protoplasts were cultured in the presence of varying concentrations of pronase and trypsin in the overlayer. At high concentrations, pronase or trypsin inhibited WLS regeneration (Fig. 8). The proteolytic activity of the pronase and trypsin solutions after 6 days in the overlayer was approximately one-half that of freshly prepared enzyme solution, presumably due to diffusion of enzyme into the underlayer. When 1% bovine serum albumin was included in the ionic overlayer, there was no inhibitory effect on wall regeneration, eliminating the possibility of a general inhibitory effect of proteins on WLS formation. Since the "zero" concentrations of trypsin and pronase in these experiments were in reality autoclaved samples of trypsin and pronase at the highest concentrations used (10 mg/ml), the lack of inhibition by the autoclaved enzymes is consistent with the idea that the inhibitory effects of the proteins are due to their enzymatic activity.

Effect of Auxin on WLS Formation. Auxins regulate cell wall formation (1, 3), but no reports exist on auxin effects during WLS formation. The following experiment tested whether the auxin 2,4-D had any stimulatory or inhibitory role on WLS formation. From a flask containing *Convolvulus* tissue culture cells in 250 ml of a standard liquid culture medium (with 1 μ M 2,4-D, which causes a good growth response), 6-ml samples were withdrawn and pipetted into other flasks containing 250 ml of standard liquid medium with or without auxin at 1 μ M. After 13 days in the auxin-deficient medium, the tissue had only doubled in volume and was beginning to discolor, showing signs of approaching senescence. Protoplasts were prepared from tissue grown 13 days both with and without 2,4-D, and samples from each were cultured with or without 2,4-D. Both the agar underlayer and the ionic overlayer contained 1 μ M 2,4-D when it was present. Under no condition was there any change in the incidence of WLS formation, which was between 55 and 62% in each case.

Earle and Torrey (11) showed that high auxin levels (2,4-D at 0.1 mM) were toxic to *Convolvulus* tissue culture, an observation confirmed during the present work. A direct effect on membrane permeability has been attributed to both 2,4-D and IAA, since rapid bursting of tobacco leaf protoplasts was observed in the presence of either (31). 2,4-D at 0.1 mM was therefore tested as described by Ruesink (35) for its ability to cause bursting of *Convolvulus* protoplasts in 0.5 molal mannitol. Careful counts of protoplasts remaining after 1 hr showed emphatically that auxin did not produce the rapid bursting previously described for onion root, tomato root, and tobacco leaf protoplasts (9, 28, 31). When protoplasts were cultured for 5 days under standard conditions with 0.1 mM 2,4-D, wall regeneration occurred around as many protoplasts as when they were cultured in 1 μ M 2,4-D, further evidence that high auxin

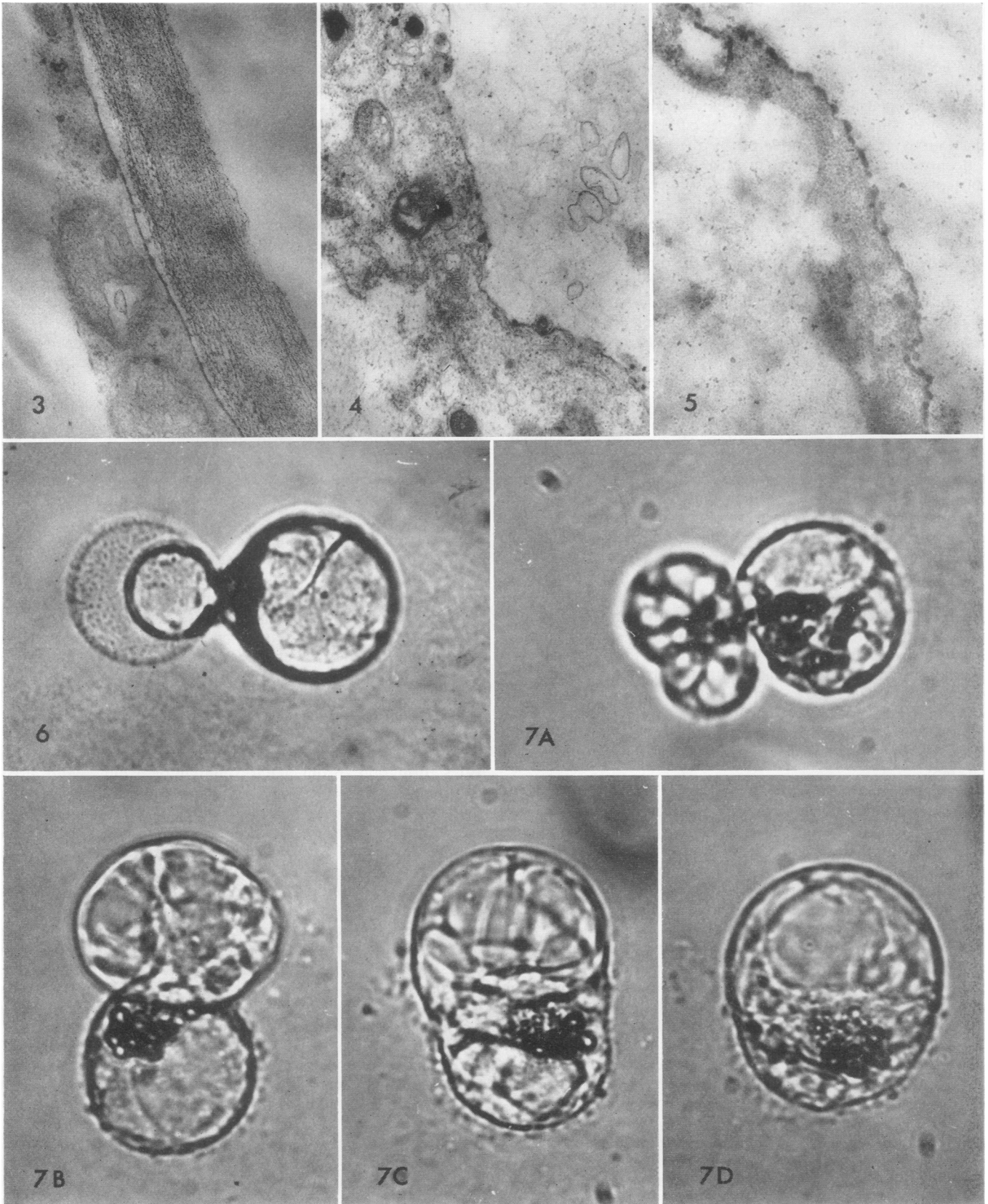


FIG. 3. Cytoplasm and wall of a typical *Convolvulus* cell at the edge of a clump of tissue. In Figures 3, 4, and 5, the plasma membrane is on the upper right hand side of the cytoplasm shown; the large central vacuole is at the lower left. $\times 34,000$.

FIG. 4. Surface membrane of a freshly isolated protoplast with a notable lack of cell wall material. $\times 27,000$.

FIG. 5. Surface membrane of a protoplast cultured 6 days under standard conditions. The electron-dense, amorphous WLS is apparent. $\times 27,000$.

FIG. 6. Protoplast that had been cultured for 6 days under standard conditions, producing a bud, before it was plasmolyzed. $\times 140$.

FIG. 7. Digestion of the WLS around a budded protoplast in the presence of 10 mg/ml *Myrothecium* cellulase. Photographs were taken (A) 4, (B) 17, (C) 26, and (D) 34 min after the addition of cellulase. The buds shown to the left in A are part of the topmost portion of the cell in B, C, and D. $\times 270$.

concentrations in this system leaves many functions at the plasma membrane unchanged. The number of protoplasts surviving in culture after 5 days in the higher auxin level was identical to the number in the lower auxin level.

Lack of Cytokinin Effects on Wall Regeneration. Inclusion of kinetin, a synthetic plant cytokinin, in the protoplast medium overlayer and underlayer was neither stimulatory nor inhibitory to wall regeneration. This was true for both concentrations of the cytokinin (1 nM, 1 μ M) tested whether in the presence or the absence of 1.0 μ M 2,4-D. Usually *Convolvulus arvensis* tissue culture is grown without any cytokinin. For several tissues (44), 2,4-D eliminates a cytokinin requirement.

Enzymic Attack upon the WLS. The ability of pectinase, pronase, and a cellulase preparation to degrade the WLS of plasmolyzed cells as revealed through the light microscope was tested. After 45 min the WLS was still intact in the presence of 50 mg/ml pectinase. Likewise, pronase at 12.5 and 25 mg/ml with the pH adjusted to 6.8 did not break down the WLS after 1 hr of application. On the other hand, a 40 mg/ml cellulase enzyme preparation from *Myrothecium verrucaria* was able to degrade the WLS completely within 30 min. Enzymes predominating in this preparation include at least two cellulases and a β -1,3-glucanase (Ruesink, unpublished). Treating the WLS for 2 hr with a β -1,3-exoglucanase (50 mg/ml) did not degrade this structure, suggesting that a callose-like molecule did not play a major role in the structure of the WLS, in agreement with the histochemical evidence reported below.

When a budding protoplast was treated with *Myrothecium* cellulase solution to remove the WLS, it was always changed back into a spherical cell (Fig. 7). This indicates that the budding phenomenon is dependent upon the regeneration of a rigid WLS around the plasma membrane. Consistent with the presence of turgor pressure in the budded cell, total volume of the cell can be seen in the figure to increase because of the osmotic uptake of water as the wall is removed. The wall around the buds is apparently digested first, since the buds expand and form a single large bud before the cell converts to a single sphere.

Test for Callose in the WLS. Plant cells sometimes produce callose in response to wounding. To test for the possible presence of callose in the WLS, protoplasts were incubated 6 days on standard medium and then washed in 0.5 molal mannitol to eliminate excess calcium. They were then placed in 0.01% aniline blue containing 0.5 molal mannitol and 70 mM K_2HPO_4 , a slight modification of the method of Eschrich and Currier (14) for detecting callose. After 15 min, the cells were observed in a Bausch and Lomb fluorescence microscope with ultraviolet illumination and a yellow filter. No fluorescence could be detected from the WLS, although sieve tubes in stems of grape and kidney bean treated in parallel showed abundant fluorescence. This test, in conjunction with the resistance to β -1,3-glucanase digestion mentioned above, indicates that the WLS does not contain appreciable callose.

Protoplasts from Crown Gall and Soybean Tissue Cultures. The system for obtaining wall regeneration around *Convolvulus* tissue culture protoplasts was used for culturing protoplasts from both crown gall tumor and soybean tissue cultures. Protoplasts from these tissues were obtained in large numbers after digestion for 2 hr with *Myrothecium* cellulase. The size of crown gall protoplasts was generally from 70 to 120 μ in diameter, while soybean (*Glycine max* L. Merrill var. Acme) tissue culture protoplasts were approximately 30 to 50 μ in diameter. A very small percentage (less than 3%) of the crown gall protoplasts underwent budding, and no soybean protoplasts displayed this activity. Upon plasmolysis, a detectable WLS could be observed around only a small fraction (less than

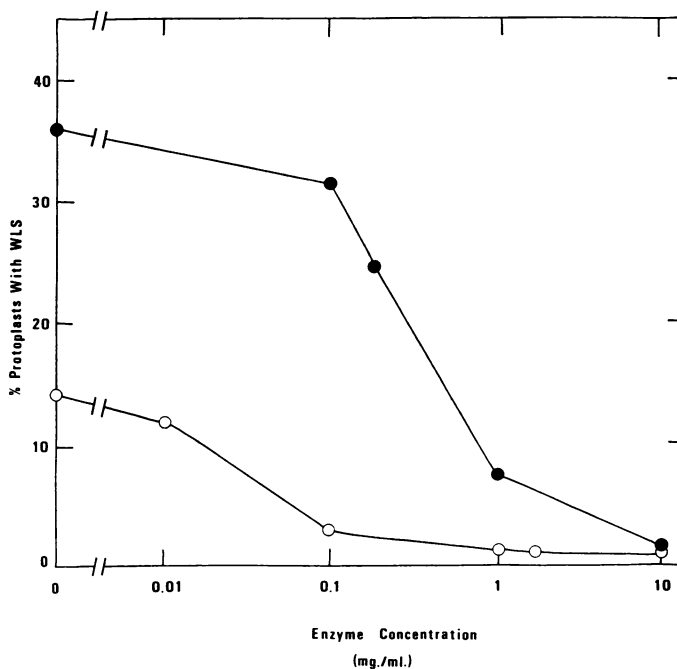


FIG. 8. Inhibitory effect of proteolytic enzymes on WLS regeneration of protoplasts from *Convolvulus*. Protoplasts were cultured on ionic medium for 4 days in the presence of pronase (●) and transferred onto standard medium for 5 days. Trypsin (○) was added to the standard overlayer in 50 mM tris at pH 7.1 and protoplasts were cultured under standard conditions for 6 days. Zero concentrations of the enzymes were autoclaved samples of each enzyme at 10 mg/ml. Each point represents an average of two experiments with two dishes per concentration per experiment.

1%) of the crown gall protoplasts, and no detectable WLS was seen around the soybean protoplasts. Therefore, these cells require something for initiations of wall regeneration that is not present in the medium used for *Convolvulus* protoplasts. Cell division and wall regeneration for soybean protoplasts cultured in conditioned medium have been reported (20).

DISCUSSION

Although no one has rigorously proved that higher plant protoplasts are as devoid of wall as bacterial protoplasts (7), several lines of evidence indicate that the amount of wall around the protoplasts used for these studies is, at most, vanishingly small. (a) Cells of all shapes assume a spherical shape as a protoplast. (b) Protoplasts are extremely sensitive to osmotic shock and other physical disturbances. (c) No residual wall is visible in light or electron microscopes. (d) At the start of the wall removal process, large regions of the protoplast surface plasmolyze away from the apparently solid cell wall. The plasma membrane-cell wall interface is by far the most likely place for this to occur.

When this work was initiated, the dramatic budding phenomenon was utilized as an indication of wall regeneration. Later it was found that the WLS of plasmolyzed cells could be directly observed easily in the light microscope and subsequent work was assayed by this method. Kao *et al.* (20) suggested that the budding of protoplasts seen by several investigators indicated regeneration, but this relationship until now had not been thoroughly investigated. Results reported here confirm that a budding protoplast is a protoplast which has regenerated a WLS upon its surface. On the other hand, to score only budding protoplasts from a population as an indication of the

number which have undergone regeneration would eliminate those cells that have undergone regeneration but have not budded. Very rapid wall regeneration would produce walls strong enough to resist all tendency for budding. In the present work, this occurred to some of the protoplasts placed onto a sucrose underlayer after 5 days on an ionic underlayer. The relationship between budding and wall regeneration can be stated as follows: protoplasts regenerating a WLS usually, but not always bud, while protoplasts not regenerating a WLS never bud. Some of the points in Figure 2 seem to dispute this by indicating a greater percentage budding than exhibiting WLS. This means that early stages of WLS formation provide sufficient rigidity to induce budding, but are not sufficiently thick to be recognized in the light microscope around plasmolyzed cytoplasm as a WLS.

Although the use of a bilayer culturing system introduces some uncertainty as to the exact concentration of molecules surrounding the protoplasts at any given time, the system does provide certain important advantages. Placed directly into 0.5 molal sucrose, *Convolvulus* protoplasts float and are rapidly broken at the air-water interface. In the standard ionic osmoticum of the overlayer, the protoplasts sink and end up in a flat plane on the agar where they can be easily observed microscopically. The underlayer provides an easily manipulated repository of nutrients that can diffuse into the overlayer and be utilized by the protoplasts. Protoplasts must be moderately close to the surface of the medium to get sufficient aeration without agitation since large vacuolated protoplasts are lysed by shaking. The bilayer system places them approximately 0.5 mm from the surface where oxygen needs can be readily met by diffusion, yet in a total of 6 ml of medium, which minimizes changes in osmotic concentration due to evaporation or condensation.

In contrast to the long term survival of ionic cultured protoplasts, where as many as 1500 to 2000 protoplasts are present, as few as 5% or less of that number sometimes survive 15 days culture under standard conditions. This finding is of interest since it is the same condition that stimulates wall regeneration. Metabolic activity is obviously occurring as evidenced by the regeneration of new wall material. Nevertheless, the optimum condition for synthesis is not the optimum condition for long term survival. Lysis could be resulting from an accumulation of some toxic product of metabolism. Another possibility could be that osmotic swelling and subsequent lysis are due to sugar or ion uptake, or both by the protoplast, although the protoplasts cultured in the presence of sucrose where survival is poorest have smaller diameters than protoplasts cultured under ionic conditions, where survival is good. The need for a divalent cation to maintain plasma membrane integrity (35) is not involved, since a minimum of 16 mM calcium is present under standard conditions. Most certainly, if new plants are ever to be routinely attained from protoplasts, a better understanding must be reached of the many effects which conditions conducive for wall regeneration have on protoplasts.

The percentage of protoplasts exhibiting WLS formation when cultured under standard conditions varies with each experiment, although replicates in a given experiment are always close to each other. The reason for this variability is not entirely understood, although it depends to some extent on the condition of the *Convolvulus* callus and to a larger extent on the particular batch of cellulase used in preparing the protoplasts.

Using the anthrone assay (38) for total sugars and the Glucostat assay for glucose (following methods provided by Worthington Biochemicals with their reagents), it was found that under standard conditions the amount of sucrose found

in the ionic overlayer reaches a relatively high amount, approximately 0.2 M, after only 1 day of culture. At the same time, glucose is present in the medium, but at a much lower concentration, about 3 mM. The glucose concentration remains constant with time, while the sucrose concentration rises slowly. There is no evidence showing whether both or only one of the sugars is utilized for WLS formation. Glucose alone cannot be used as the osmoticum, since *Convolvulus* protoplasts are unstable in it, as are *Avena* coleoptile protoplasts (36).

The lack of any noticeable effect of actinomycin D, puromycin, or cycloheximide on WLS regeneration suggests that no major synthesis of RNA or of protein after protoplast formation is required for WLS production. *In vitro* systems of various polysaccharide synthetases are quite labile, being rapidly inactivated with mild changes in pH or increases in temperature (5, 41, 42). Although the rate of turnover of these enzymes *in vivo* is not documented, their behavior *in vitro* suggests that it might be quite rapid. Since the WLS seems to be carbohydrate, the enzymes that synthesize the WLS are presumably identical to some of the ones which synthesize the native cell wall and should disappear quickly in the absence of protein synthesis. The effect on protein synthesis in the intact tissue by these inhibitors was not one of complete inhibition. Therefore, the residual protein synthesis may be sufficient for activation of regeneration. Yeast protoplasts, in contrast to the present results, produce the fibrillar components of the regenerated wall, but not the amorphous matrix when treated with cycloheximide (26).

Eddy and Williamson (12) with yeast and Mishra and Colvin (22) with tomato fruit protoplasts reported that the regenerated WLS was not similar to the original cell wall. Though the chemical nature of the WLS around *Convolvulus* protoplasts is not known at present, the electron micrographs show it is amorphous and lacks the fibrillar nature of the native cell wall. This is in direct contrast to reports that the regenerated walls around tomato fruit protoplasts (22, 29) and onion root protoplasts (33) do contain fibrillar components and indicates, nor surprisingly, that different kinds of protoplasts will produce different kinds of WLS. The *Convolvulus* WLS is susceptible to cellulase attack; therefore the lack of fibrillar components in it does not reflect just the presence of residual cellulase in the isolation medium, although, to be sure, the tomato fruit protoplasts, which did regenerate fibrils, were isolated with pectinase alone.

The effects of proteolytic enzymes have been better documented on mammalian membranes than on plant membranes. The widespread use of proteases for mammalian tissue dissociation causes specific injury to the cell surfaces (18, 27, 37). Trypsin and pronase adsorb to the surfaces of cells dissociated from dog kidney tissue and prevent the formation of cell coat material (30). Similarly, proteolytic enzyme action at the membrane surface of *Convolvulus* protoplasts has an inhibitory effect on wall regeneration; however, the concentrations of the proteolytic enzymes required for inhibition of WLS regeneration are quite large, indicating considerable protection of the proteins. Probably these enzymes degrade a protein or proteins located on or somewhat buried in the plasmalemma that are required for the synthesis of the WLS. Previous work has shown that pronase does not appreciably alter the stability of higher plant protoplasts (34, 36). That pronase is not altering the WLS itself is shown by the lack of effect that pronase has on budded protoplasts and the inability of pronase to enhance the digestion of the original wall (Ruesink, unpublished).

The synthesis of a WLS by *Convolvulus* protoplasts prepared from tissue depleted of most, if not all, of their 2,4-D, is not affected by the level of 2,4-D in the culture medium. The con-

clusions about auxin effects here are based on data which describe the number of cells undergoing wall regeneration in the presence or absence of auxin. There are not data on whether any difference exists in the amount or nature of the regenerated WLS when protoplasts are cultured either with or without auxin. Thus it is not possible to conclude from these data that auxin does not stimulate WLS synthesis quantitatively by facilitating RNA or protein synthesis. However, the results from the RNA and protein synthesis inhibitor studies discount any major involvement of RNA and protein synthesis as necessary for stimulating WLS synthesis.

The studies reported here indicate that protoplasts isolated by cellulase treatment will indeed form some sort of wall during subsequent culture. Preliminary experiments to get the protoplasts with regenerated walls to divide, grow, and form clones were unsuccessful. Unfortunately, it is thus impossible to state how pertinent the WLS formation is to a viable situation. WLS formation is, of course, a metabolic process regulated by the protoplast in response to external factors. A number of ways of controlling whether or not a WLS will form have been described. As techniques are developed for analyzing the nature of the WLS produced, it should be possible to change the character of the WLS formed and get walls of desired structure for various purposes.

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