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Review of Cytology

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Protoplasts of Eukaryotic Algae

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I. Introduction

A. Objectives

Protoplasts of higher plants have become important experimental tools in the past decade (Cocking, 1980), whereas protoplasts of eukaryotic algae, which were first reported in 1970 by both Chardard and Gabriel, have not received the attention they deserve as potential models for many current aspects of cellular research. Stewart's book (1974) on algal physiology and biochemistry does not mention the word protoplast, and neither does Pickett-Heaps' (1975) compendium on the green algae. The literature on algal protoplasts through 1977 has been reviewed by Adamich and Hemmingsen (1980) but is limited to protoplast production methods without discussion of experimental work and of their future in research in areas such as somatic hybridization, morphogenesis, and organelle structure and function.

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This article proposes to bring information on protoplast induction up to date and to discuss the advantages and problems of working with protoplasts of eukaryotic algae.

B. Definition of Terms

Within the framework of this review, the term *protoplast* is defined as a viable cell whose wall and other materials external to the plasmalemma have been removed, but which retains all internal components (Adamich and Hemmingsen, 1980). Additional criteria are (*a*) spherical shape, usually of greater volume than the vegetative cell, (*b*) osmotic fragility, (*c*) physiological activity, (*d*) internal structural integrity, and (*e*) the potential for cell wall regeneration and cell division. Physiological activity and internal structure need not necessarily be similar to that of intact cells as required by Adamich and Hemmingsen (1980), and regeneration and division are frequently difficult to ascertain.

C. Scope

This article is limited to eukaryotic algae, since they most closely parallel the extensive work on vascular plant protoplasts and have little relationship to protoplast studies with prokaryotic blue green algae (Adamich and Hemmingsen, 1980). In addition, this article will not consider what have been described as "wall-less" algae such as *Dunaliella* (Marano, 1976), *Asteromonas* (Floyd, 1978), and *Oftmansiella* (Hargraves and Steele, 1980), because glycoprotein wall materials have recently been demonstrated for these types of organisms by improved cytochemical methods (Oliveira *et al.*, 1980), and also because these cells are neither affected by hydrolytic enzymes nor are they osmotically fragile (Berliner, 1981). Wall-less cells occurring as transient stages as in *Chlamydomonas* gametogenesis are also not included (Matsuda, 1980).

II. Protoplast Methodology

A. Organisms

The review by Adamich and Hemmingsen (1980) lists the following genera of eukaryotic algae (Stewart, 1974) for which protoplast induction has been reported: Chlorophyceae—*Chlamydomonas, Chlorella, Cosmarium, Klebsormidium, Micrasterias, Mougeotia, Spirogyra, Stigeoclonium, Ulothrix, Zygnema*. Bacillariophyceae—*Nitzschia*. Pyrrophyceae—*Gonyaulax*. Rhodophyceae—*Porphyridium*.

Subsequent reports of other true protoplast obtention from additional genera

are: Chlorophyceae—*Boergesenia* (Kosuge and Tazawa, 1979), *Chlorosarcinopsis* (Berliner, 1981), *Cladophora* (Berliner, 1981), *Closterium* (Berliner, 1978), *Cylindrocystis* (Berliner, 1981), *Derbesia* (Wheeler and Page, 1980), *Enteromorpha* (Millner *et al.*, 1979), *Fritschiella, Mesotaenium* (Berliner, 1981), *Netrium* (Berliner, 1978), *Nitella* (Abe *et al.*, 1980; Kuroda, 1980), *Oedogonium* (Berliner, 1981), *Polyphysa* (*Acetabularia*) (Zimmer and Werz, 1980), and *Staurastrum* (Berliner, 1978). Haptophyceae—*Hymenomonas* (Safa-Esfahani, 1980).

All are fresh-water organisms except for *Boergesenia* (Kosuge and Tazawa, 1979), *Chlorosarcinopsis* (Berliner, 1981), *Enteromorpha* (Millner *et al.*, 1979), *Gonyaulax* (Adamich and Sweeney, 1976), *Hymenomonas* (Safa-Esfahani, 1980), *Polyphysa* (*Acetabularia*) (Zimmer and Werz, 1980), and *Porphyridium* (Clement-Metral, 1976). All these organisms are either unicellular or uniseriate filaments except for *Enteromorpha* (Millner *et al.*, 1979) which forms a macroscopic tubular thallus.

Usually little has been known about the structure and chemical composition of the cell walls and mucilagenous matrices that envelop the genera and species of algal cells used for protoplast induction. One of the exceptions was the deliberate selection by Braun and Aach (1975) of *Chlorella* species whose walls did not contain sporopollenin.

All reports of successful protoplast obtention were from mature vegetative cells except for *Derbesia* maturing gametophytes (Wheeler and Page, 1980) and for gametes in *Chlamydomonas* (Schlosser *et al.*, 1976). Attempts to obtain protoplasts from zygotes of *Closterium* and *Cosmarium* were not successful (Berliner, 1981).

B. Culture Conditions

Algae from which protoplasts will be induced have always been grown as liquid cultures, but there is no uniformity among the media and all are chemically undefined, containing either seawater or soil extract, and sterilized by filtration or autoclaving. The pH is often not adjusted nor monitored during protoplast formation. All commonly used enzymes and most osmotica such as mannitol substantially lower the pH in the range of 4.0–5.5.

Whether or not the algae were axenic is often not mentioned, and both bacteria-free and contaminated cultures have been used in obtaining protoplasts. Rendering algal cultures axenic is often difficult because the mucilagenous slime that naturally surrounds many algal species is a normal habitat for bacteria. The method of Bradley and Pesano (1980) holds the most promise for bacterial removal without toxicity to the algae. Protoplasts induced from nonaxenic cultures rarely survive because the bacteria thrive on the enzyme and the osmoticum.

The optimal physiological state of the alga prior to protoplast induction has often not been ascertained. Where this has been studied, it has generally been found that cultures in log phase (Berliner, 1977), just prior to division (Marchant and Fowke, 1977) or just after division (Berliner and Wenc, 1976a,b), yield the largest number of protoplasts in the shortest time. In *Chlorella* it has been observed that the cultures must be monitored microscopically for autospores since these do not form protoplasts (Aach *et al.*, 1978; Berliner, 1977).

Light has been one of the other factors often mentioned just in passing rather than extensively studied for its effect on protoplast induction.

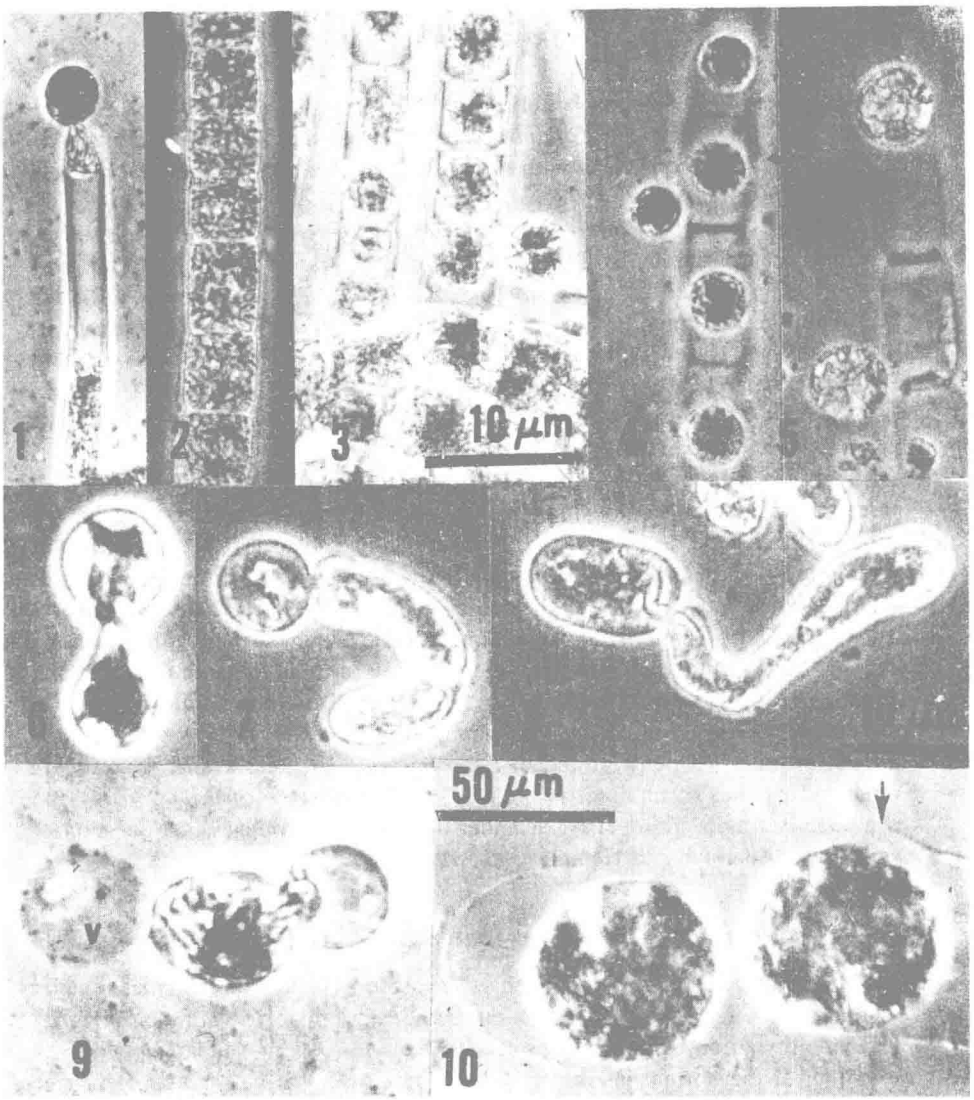
The temperature at which protoplast induction has been most successful has generally been within the normal culture range for that species, although Millner *et al.* (1979) found that a low temperature of 10–12°C was optimal for protoplast formation in *Enteromorpha*. Temperature-sensitive mutants, which form a cell wall at 25°C but not at 35°C, have been used to study cell wall development in *Chlamydomonas* (Loppes and Deltour, 1978). Hemmingsen (1971) found that protoplasts were formed by the marine diatom, *Nitzschia*, only in calcium- and/or magnesium-deficient media while Safa-Esfahani (1980) determined that Ca^{2+} was necessary for protoplast formation.

C. Enzymes and Other Methods

Although exogenous hydrolytic enzymes are the main inducers of protoplast formation, other methods have been successful. In algae that form naked zoospores, mechanical disruption of the sporangia have released these "natural" protoplasts (Kosuge and Tazawa, 1979; Robinson and Schlosser, 1978). By the same token the natural weak point at the isthmus of constricted desmids has been used to induce protoplast release by osmotic means alone (Berliner and Wenc, 1976a,b; Chardard, 1970). Detergents and high osmotica induced protoplast emergence between the theca of *Gonyaulax* (Adamich and Sweeney, 1976).

The exogenous enzymes that have been used by most investigators are unspecific mixtures of unknown exact composition that vary from lot to lot. This is true for the most common of these: Driselase, Onozuka Cellulase, Cellulysin, and Macerozyme. Snailgut juice or Helicase, which is commonly used for fungal protoplast induction, has been used only once on algae (Gabriel, 1970). All of these unpurified enzymes contain large amounts of proteases and peroxidases and desalting them, which is often done in higher plant protoplast procedures, only serves to concentrate these lytic fractions (Berliner, 1981). Commercially available enzymes are also contaminated with fungal and/or actinomycete hyphal fragments and conidia and should be sterilized by ultrafiltration.

The only specific enzymes that have been used are the autolysines of *Chlamydomonas reinhardii* (Schlosser *et al.*, 1976; Robinson and Schlosser, 1978). These enzymes are isolated from synchronized cultures of the tissue when gametes fuse and their walls are shed. This instance of the large-scale production



FIGS. 1–10. Fig. 1. *Cladophora* sp. Protoplast emerging from tip of ultimate cell of filament. Walls remain intact. Figs. 2–5. *Zygnema* sp. protoplast formation. Fig. 2. Normal vegetative filament. Fig. 3. Early plasmolysis. Fig. 4. Lateral release of one protoplast per cell. Fig. 5. End of protoplast process. Side walls about to disintegrate leaving unaffected septa. Figs. 1–5. Phase contrast at magnification in Fig. 3. Figs. 6–9. *Mesotaenium caldariorum* protoplast regeneration. Phase-contrast, all at magnifications in Fig. 8. Fig. 6. Early budding with contents equally divided between the protoplast and the bud. Similar to Fig. 17. Fig. 7. Protoplast with some remaining cell content attached to regenerated filament. Fig. 8. Both original protoplast (left) and its bud have elongated and regenerated a cell wall as seen in Fig. 15. Fig. 9. Protoplast (center) forming walled bud (right) and extruding a vacuole (left). Fig. 10. *Netrium digitus*. Formation and release of two protoplasts per cell at dissolved area in side wall (arrow) with eventual disappearance of all wall material. Normarski interference phase contrast.

of protoplasts from vegetative cells by a "nonforeign" enzyme appears to be useful only for species of *Chlamydomonas* since their cell walls contain no cellulose, only glycoproteins with galactose and arabinose as the major sugars (Robinson and Schlosser, 1978).

Exogenous hydrolytic enzymes act in different ways on algal cell walls during and after protoplast release. The entire wall may be dissolved as with *Chlorosarcinopsis* (Figs. 11 and 18) and *Chlorella* (Aach *et al.*, 1978; Berliner, 1977); or a terminal pore is formed in a filamentous species such as *Cladophora* (Berliner, 1981) (Fig. 1). In other filamentous species such as *Zygnema, Mougeotia,* and *Spirogyra* (Marchant and Fowke, 1977; Ohiwa, 1977), the protoplasts are released through a lateral pore and only the tubular walls dissolve, leaving intact septa in the induction medium (Fig. 5).

In those algae that have a natural cleavage area in their cell walls, of modified or different chemical composition, this is invariably the locus through which the protoplasts emerge, as in *Gonyaulax* (Adamich and Sweeney, 1976), *Nitzschia* (Hemmingsen, 1971), *Hymenomonas* (Safa-Esfahani, 1980) and in desmids (Berliner and Wenc, 1976b). In the constricted desmids, such as *Cosmarium* and *Micrasterias,* the empty walls remain intact in the enzyme solution (Berliner and Wenc, 1976c) while in the unconstricted desmids such as *Netrium* (Berliner and Wenc, 1976b) and *Mesotaenium* (Fig. 10) the wall dissolves following protoplast release. Lastly, in *Gonyaulax* (Adamich and Sweeney, 1976) there remained a carbohydrate-rich pellicle, and in *Enteromorpha* (Millner *et al.*, 1979) there remained patches of undissolved cell wall on the surface of the spherical and osmotically fragile protoplasts. Whether these are spheroplasts is a question of semantics (Adamich and Hemmingsen, 1980).

D. Osmotica

Since induced protoplasts of fresh-water algae have lost the mechical barrier that maintains their internal osmotic pressure, they can only retain their integrity and viability in an osmotically protective medium of equal or greater tonicity than that of the normal internal cellular osmolarity. The protoplasts of marine algae such as *Chlorosarcinopsis* and *Acetabularia* (Zimmer and Werz, 1981) are osmotically fragile but do not require external osmotic protection beyond that of sea water. In only one instance (Ohiwa, 1977) has the internal osmotic pressure been measured and the information used to determine the min-

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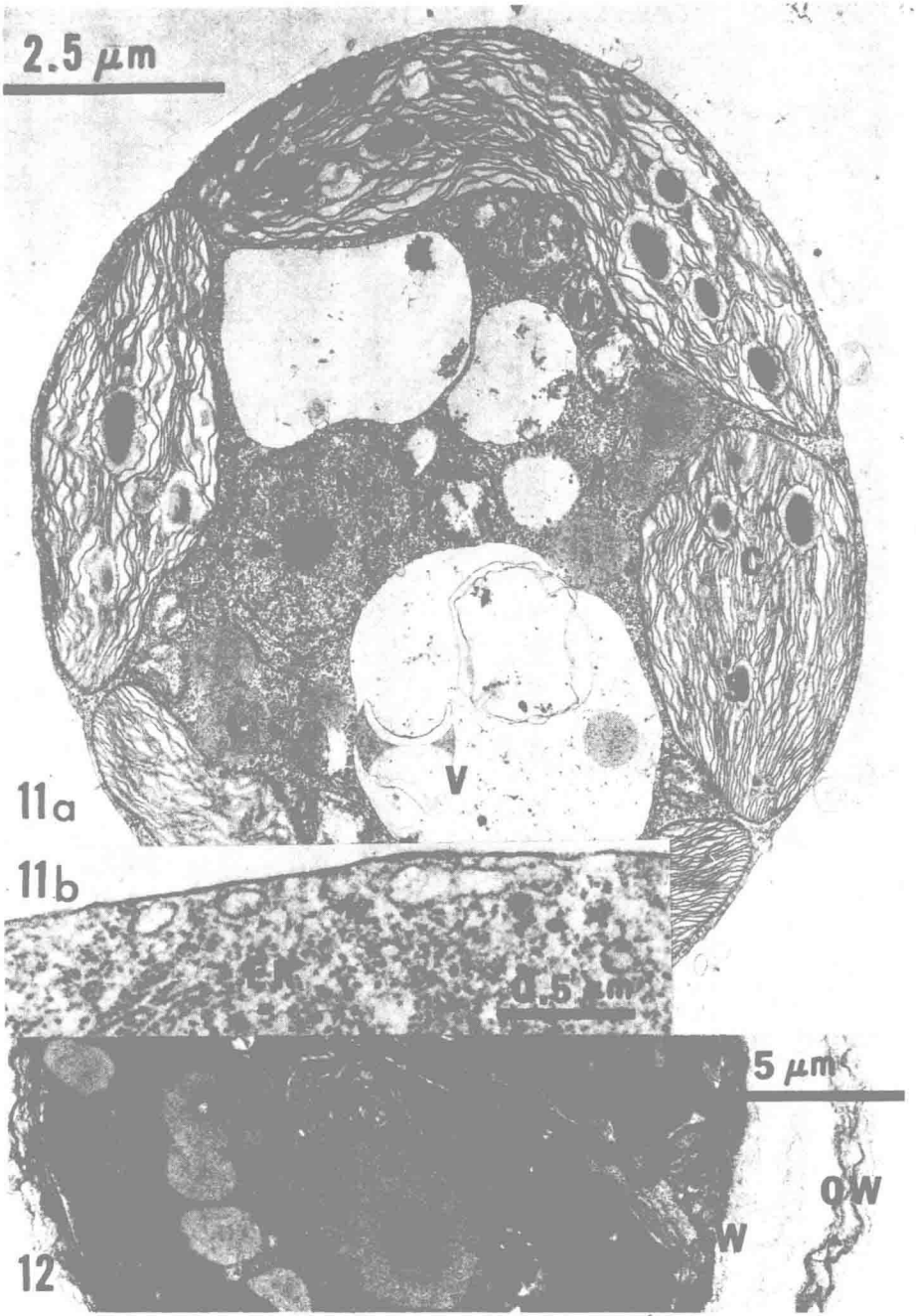
Figs. 11 and 12. *Chlorosarcinopsis halophila:* transmission electron microscopy of protoplast formation. (11a) Protoplast with well-preserved organelles. C, chloroplast; L, lipid bodies; M, mitochondria; N, nucleus with nucleolus and perforated double membrane; V, vacuole with intravacuolar membranes; S, starch. (11b) Higher magnification of plasmalemma showing total absence of wall material, ER, endoplasmic reticulum. Fig. 12. Vegetative cell showing thick fibrillar inner (IW) and outer (OW) walls, which interfere with successful fixation of organelles.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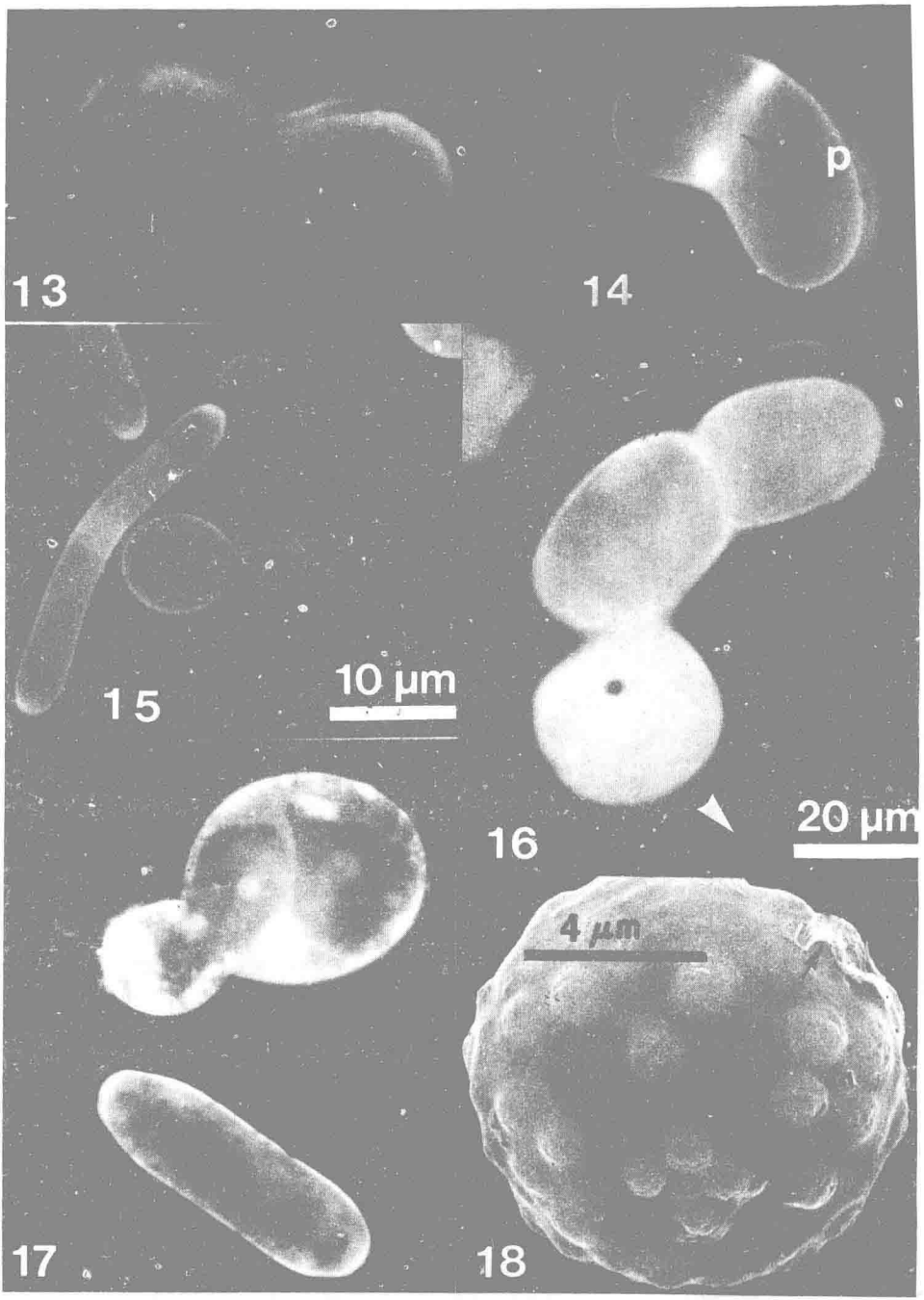
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imal hypertonicity needed to protect protoplasts. The internal osmotic pressure of *Spirogyra* was 0.38 *M* sucrose-equivalent and that of *Zygnema* was 0.27 *M* sucrose-equivalent. The surface tension on the giant protoplasts of *Nitella* in nonenzyme medium was 0.2 dyne/cm^{-1} or equivalent to that of mature sea urchin eggs (Kuroda, 1980).

The substances most commonly used as osmotica in all plant protoplast work, including algae, are mannitol, sorbitol, $MgSO_4$ and KCl, either alone or in combination. These substances are rarely metabolized and often do not support the growth of contaminants. Several studies on the effective molar range of these substances have been done (Berliner and Wenc, 1976a; Chardard, 1972; and Marchant and Fowke, 1977) and have generally concluded that mannitol and sorbitol are the least toxic and generally most effective in a range of 0.3 to 0.5 *M* mannitol or equivalent. Only for the large thallus of *Enteromorpha* (Millner *et al.*, 1979) was a 1.2 *M* concentration of sorbitol required to induce and maintain protoplasts.

The cells of fresh-water algae are invariably plasmolyzed in the effective osmotica, and this plasmolysis is often the best indicator of the preprotoplast stage (Berliner and Wenc, 1976a). On the other hand, marine algae such as *Gonyaulax* (Adamich and Sweeney, 1976), *Chlorosarcinopsis* (Berliner, 1981), *Enteromorpha* (Millner *et al.*, 1979), and *Nitella* (Abe *et al.*, 1980) usually are not plasmolyzed at any hypertonicities prior to protoplast release. Some researchers have preplasmolyzed cells in osmoticum prior to adding the enzyme (Berliner and Wenc, 1976a, b; Ohiwa, 1978) to try to shorten the time that cells need to be exposed to the enzyme, but with little success. Ohiwa (1978) did establish that only protoplasts of *Spirogyra* and *Zygnema* that had been preincubated in the osmoticum had the potential for completing protoplast fusion.

E. Separation

There are few details available on the separation of protoplasts from the inducing medium. Removal of the enzyme and maintenance of the osmoticum are imperative in preserving morphologically and physiologically intact proto-

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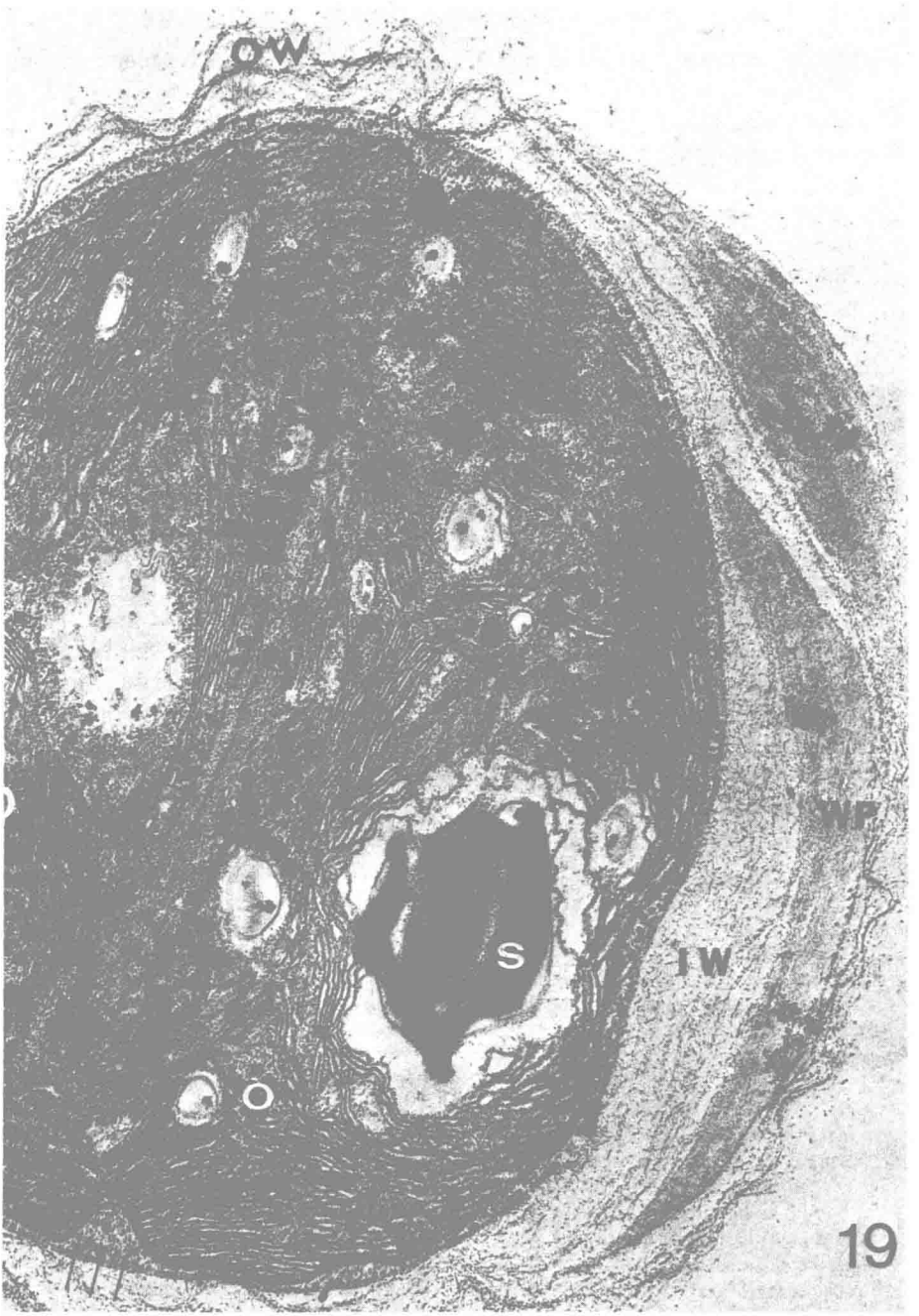
FIGS. 13–17. Cell wall regeneration in protoplasts of *Mesotaenium caldariorum*. Magnification in Fig. 15 applies to Figs. 13, 14, and 17 as well. Figs. 13–16, ultraviolet microscopy of Calcofluor-stained cells. Fig. 13. Elongated protoplast with multilayered accumulation of amorphous wall material not visible in phase-contrast microscopy as in Fig. 8. Protoplast still osmotically fragile. Fig. 14. Early septum formation and amorphous wall deposition. Fig. 15. Protoplast bud elongation into filament of diameter similar to that of control cell on the left. Both the protoplast and the filament have Calcofluor-positive walls. Fig. 16. Short and stubby regenerated protoplast as in Fig. 14, which has divided and is shedding Calcofluor-positive primary wall (arrow). Fig. 17. Dark-filled light microscopy of early budding protoplast and normal cell. Cell contents are outlined but cell wall deposition is not visible.

FIG. 18. Scanning electron microscopy of *Chlorosarcinopsis halophila* protoplast with conspicuous lipid globules just beneath the torn plasma membrane (Fig. 11a) (arrow).



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plasts that will have the potential to regenerate. Both Millner *et al.* (1979) and Adamich and Sweeney (1979) used a Ficoll gradient, while the latter and Berliner (1981) (Fig. 20) have provided detailed flow diagrams of separation techniques.

F. Viability

Protoplast viability has most commonly been assessed by the exclusion of vital dyes by living cells (Berliner *et al.*, 1978). The dyes most commonly used are Trypan Blue (Berliner *et al.*, 1978), Crystal Violet (Clement-Metral, 1976), Neutral Red (Millner *et al.*, 1979), and Fluorescein Diacetate (Marchant and Fowke, 1977; Safa-Esfahani, 1980). The latter author also correlated viability to counts of cells that regenerated flagellae and became motile. Colony counts of regenerated cells in soft agar were done by Braun and Aach (1975). The last two methods measure regeneration potential rather than protoplast viability.

G. Regeneration

Regeneration of an outer wall followed by cell division is the desired outcome of most protoplast experimental work. Its successful achievement has eluded many investigators and the various methods that have been described in any detail are all derived from experience with higher plant protoplasts. The modifications that have succeeded have little rational basis and are most often the results of chance or guesswork. The flow diagram for regeneration of protoplasts of *Mesotaenium* (Fig. 20) incorporates most of the factors that must be considered and modified for each organism. These are: (1) the pH which universally needs to be raised as soon as enzyme is removed; (2) the osmoticum which must be decreased as soon as a cell wall matrix is laid down—this to be checked by using Calcofluor fluorescence in ultraviolet light of polysaccharide materials; (3) Ca^{2+} ions to stabilize the naked plasmalemma; (4) diffuse or no light; (5) temperature usually slightly higher than for normal growth; (6) shaking and aeration which although often tried seem to make little difference. All detailed reports of regeneration and cytokinesis have considered these criteria (Berliner, 1981; Marchant and Fowke, 1977; Ohiwa, 1978, 1980; Safa-Esfahani, 1980).

Fig. 19. *Chlorosarcinopsis halophila* protoplast regeneration after 72 hours. The outer wall (OW) has the typical wavy appearance of the control vegetative cells (Fig. 12). Oblique sections through the inner wall (IW) show both granular and fibrillar regions. The wall patches (WP) are temporary and disappear after the first division. The single chloroplast is well-developed with many osmiophilic droplets and some starch. The double arrows in the center point to spindle-organizing microtubules. Triple arrow in lower left point to the early cytoplasmic invagination preceding cytokinesis and cross-wall formation. C, Chloroplast; O, osmiophilic granules; Ve, vesicles; S, starch.