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Cell Wall Synthesis in Yeast Protoplasts

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INTRODUCTION

Although many observations in older cytological literature prove that the naked protoplasts of different lower and higher plant cells are able to synthesize new cell walls, only the large scale isolation of protoplasts has enabled a detailed analysis of this process. A large amount of yeast protoplasts can be prepared by controlled autolysis of the cell wall in *Saccharomyces cerevisiae* (62) and other budding yeasts (45). Eddy and Williamson (14), starting from the observation of Giaja (30) that the gut juice of the snail *Helix pomatia* dissolved the cell walls of yeast cells, elaborated an excellent, routine method for the preparation of protoplasts. Digestive enzymes from the stomach of *H. pomatia* (snail enzymes) can be used for isolation of protoplasts in nearly all yeast species.

The regeneration of yeast protoplasts to normal cells was described first in *S. cerevisiae* protoplasts prepared by autolysis of the cell wall (62, 63). Eddy and Williamson (15) observed the formation of aberrant cell walls by growing protoplasts of *S. carlsbergensis* in liquid nutrient media. A quantitative reversion of protoplasts to normal walled cells was found after embedding the protoplasts into gel media (69). Since that time, knowledge regarding the biosynthesis of the cell wall in yeast protoplasts has increased steadily.

Protoplasts of all yeast species studied so far are able to regenerate new cell walls and revert to normal reproducing cells. Thus, in yeast protoplasts we have a unique opportunity to study all steps of the biosynthesis of the cell wall. Moreover it is possible, by changing the cultivation

conditions or by the use of inhibitors, to uncouple the formation of the fibrillar groundwork of the wall from that of the wall matrix and to study both processes separately. Finally, these experiments can lead to significant conclusions about the role of the cell wall in cell morphogenesis.

ARE YEAST PROTOPLASTS TRUE PROTOPLASTS?

In bacteria, a distinct line is drawn between protoplasts and spheroplasts. The former are completely deprived of the cell wall; the latter are enveloped by wall remnants (6). For a long time, bacterial protoplasts (contrary to bacterial spheroplasts) were believed to lose the capacity to regenerate a new cell wall, and the wall residues in spheroplasts have been considered a necessary primer for the formation of a new bacterial cell wall. Regarding this view, generally accepted among bacteriologists, there has been frequent discussion of whether the regenerating yeast protoplasts are true protoplasts or formations of the spheroplast type. Many authors have made efforts to prove wall remnants in yeast protoplasts, and believed that they found them; others produced evidence that only the plasma membrane forms the protoplast surface. Thus, a polemic has arisen between two camps and it continues to the present time. This dilemma has also been reflected in the terminology, exactly the same formations being termed yeast protoplasts, "protoplasts," protoplast-like bodies, and spheroplasts.

This question is, undoubtedly, very important when considering the information controlling the architecture of the cell wall during its formation. In other words, does the cell wall represent an autoreproductive cell structure (omnis structura e structura) or can it originate de novo without any continuity with the existing structure or prestructure? Therefore, it seems necessary to collect the most important data supporting both views and to attempt to find an approximate conclusion.

Chemical Analysis

Direct chemical analysis of fractionated protoplasts can hardly lead to the solution of this problem. First, the cell wall in yeast is composed of glucans, mannans, proteins, and lipids, all these constituents being also present inside the cell. No specific chemical markers (e.g., comparable to diaminopimelic acid in bacteria) have been found in yeast cell walls. Second, the protoplast suspension is always contaminated with free, incompletely digested cell walls and particularly with bud scars which are extremely resistant to attack by snail enzymes (3). The positive serological proof of wall material in protoplasts of *Candida utilis* (29) may, therefore, be caused by contamination with detached wall residues. Streiblová et al. (99) found no primuline fluorescence of *S. cerevisiae* protoplasts. (Primuline is specifically bound to the cell wall structure.)

Kinetics of Protoplast Formation

A gradual degradation of the yeast cell wall starts after the addition of wall lytic enzymes. Thus, during the preparation of protoplasts, a spheroplast state must logically be anticipated. Darling et al. (10) used the term prospheroplast for the transient state in which the yeast cell, treated with snail enzymes, becomes osmotically sensitive but retains a rigid coating and the shape of the original cell. The question is, however, whether the spheroplast state is transient or remains till the final stage of the preparation. According to the observations made by most authors, either complete digestion of the cell wall takes place, especially after prolonged action of lytic enzymes (28), or, more frequently, the protoplasts are liberated from the incompletely digested wall remnants (28, 56, 71, 81, 89, 113). The escape of protoplasts from the wall remnants is probably caused by changing osmotic conditions and by surface-tension forces (88). It can happen, however, that the liberation of protoplasts does not take place in some cases, and consequently some spheroplasts can be present in a protoplast population (10, 86). It may depend on the different sensitivity of the yeast strains to the lytic enzymes and on the different sensitivity of the individual cells, the walls of older cells being highly resistant (2, 14, 27, 58).

Electron Microscopy of Protoplasts

The remnants of original cell walls enveloping the protoplasts, if present, should be observed by means of the electron microscope after osmotic lysis of protoplasts. Since wall remnants are always present in the protoplast population (3, 10, 71), only observation of protoplast lysis in situ can bring some information. These studies (71) have shown, however, that for the most part only clumps of disrupted plasma membrane can be found besides other cell structures such as tonoplast membranes, nuclear envelopes, etc. Moreover, although the number of disrupted protoplasts on the supporting membrane of the electron microscopy grids was great, only few wall residues were present with or without contact with individual lysed protoplasts.

All papers dealing with electron microscopy of yeast protoplasts on ultrathin sections reported only the presence of plasma membrane on the

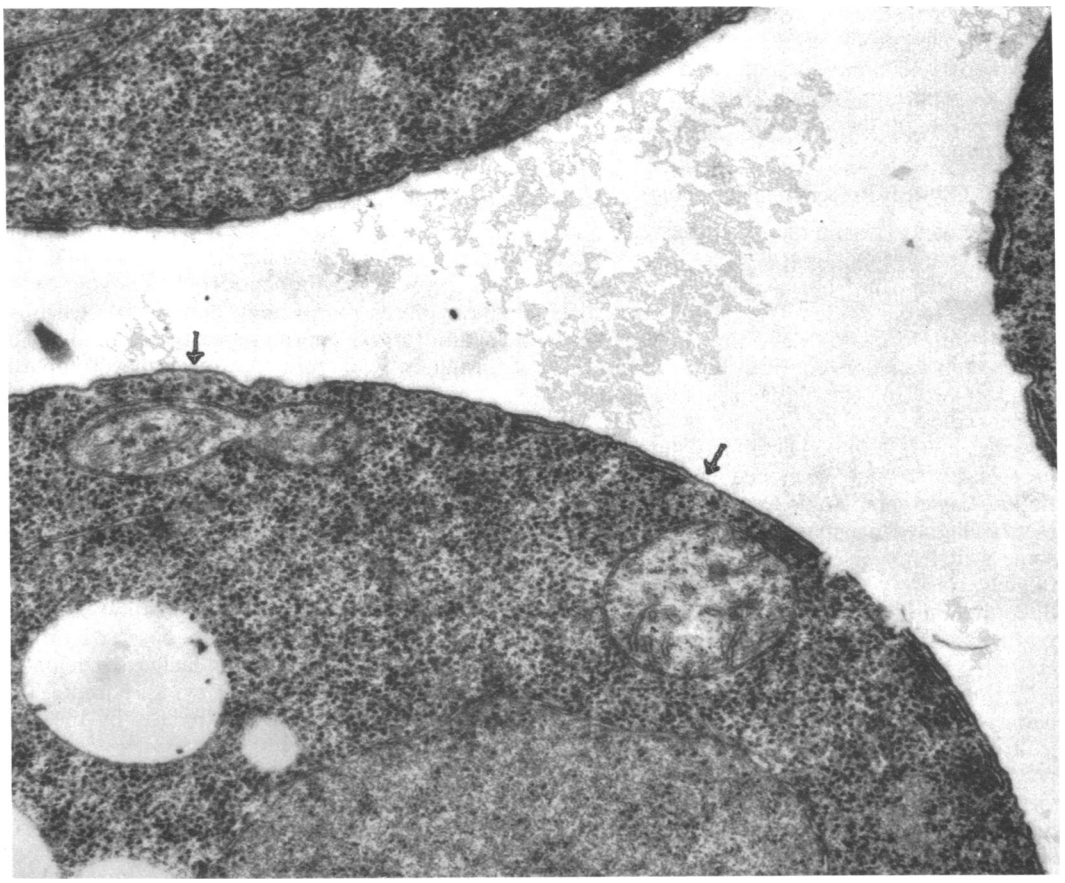

FIG. 1. *Ultrathin section of protoplasts of Saccharomyces cerevisiae. Only the plasma membrane (a unit membrane) can be seen on the protoplast surface (arrows). Mitochondria, cisternae of endoplasmic reticulum, vacuoles, and numerous ribosomes inside the protoplasts. Fixation with glutaraldehyde-osmium tetroxide; post-fixation with uranylacetate.* × *40,000. (Courtesy of Marie Havelková.)*

protoplast surface: in *S. carlsbergensis* (17, 32), *S. cerevisiae* (40, 41, 71, 73), *Endomyces magnusii* (56, 114), *C. utilis* (32, 113), *S. lactis*, *Saccharomycodes ludwigii* (38), *Lipomyces lipofer* (38), and *Nadsonia elongata* (32). The various fixatives and embedding materials used in the papers cited above did not reveal any differences to the structure shown in Fig. 1. Only in protoplasts of *Saccharomyces cerevisiae*, prepared by autolysis of the cell wall, were incompletely digested walls enveloping the protoplasts occasionally found in ultrathin sections (71). In prospheroplasts, the wall remnants were clearly demonstrated (10).

The freeze-etching method might be looked upon as an ideal tool for answering the question as to whether yeast protoplasts are true protoplasts or whether some structural remnants of the original cell wall are present on their surface. The results published indicate, however, that an interpretation of the structures found in the replicas is not as easy as one would expect.

Streiblová (97) described a thin, nonetchable layer adhering to the plasma membrane in some protoplasts. She suggested that this layer corresponded to the plastic innermost layer of the original cell wall, probably containing proteins. Others (79) invariably found this nonetchable layer adhering to the protoplast surface. Many facts, however, indicate that this layer is identical to the eutectic layers which arise upon freezing of a protoplast suspension. Theoretically, there is no possibility of avoiding these artefacts because when suspending the protoplasts in solutions maintaining stable osmotic conditions, we have to disregard their eutectic composition. Moreover, this layer does not disappear after prolonged treatment with snail enzymes and proteolytic enzymes such as trypsin and Pronase (80).

A similar nonetchable layer was found on other kinds of cells (protozoa, red cell ghosts, etc.) prepared by the freeze-etching technique. Some authors believe that this layer is identical to the outer leaflet of the split-unit membrane of the plasma membrane.

Physical Properties of Protoplasts

The spherical shape of protoplasts can be reversibly changed by physical forces, as can be observed under the phase-contrast microscope. Capillary forces flatten the protoplasts to extremely thin forms (68), and thus the surface-to-volume ratio changes considerably. The measurements of swollen protoplasts in slightly hypotonic media indicate that the increase in surface area may reach 300% (66). These facts show that the protoplasts are not enveloped by a rigid, continuous layer comparable to remnants of incompletely digested original cell walls. If there are some wall remnants, they would have to be extremely elastic or cover the protoplast surface discontinuously, making islands of wall material.

Conclusion

Evaluation of these facts, based on the dynamics of protoplast formation, light and electron microscopic morphology, and the physical properties of protoplasts, strongly supports the view that, at least in the majority of cases, the protoplasts have completely lost the original cell wall. A contamination of the protoplast population with a small number of spheroplasts, which can never be excluded, is not decisive for the problem in question. After embedding protoplasts in solid gel media, as will be shown later, they can revert quantitatively to normal cells; i.e., practically all protoplasts can regenerate new cell walls. Thus the wall remnants, even when occasionally present on the protoplast surface, cannot represent a necessary primer for the biosynthesis of the yeast cell wall.

CELL WALL SYNTHESIS IN S. CEREVISIAE PROTOPLASTS

Basic information concerning the wall biosynthesis in yeast protoplasts has been collected in budding yeasts, especially in *S. cerevisiae*. Therefore, it seems useful to describe in detail the wall regeneration in this model system first and to later describe the wall regeneration in other yeasts, whenever it differs from that of *S. cerevisiae*.

Cultivation of Protoplasts in Liquid and Solid Media

There is compelling evidence in the literature that the synthetic capacity of yeast protoplasts is not substantially affected when compared with normal walled cells. They grow in osmotically stabilized nutrient media, growth being accompanied by repeated nuclear division (64, 68). Synthesis of nucleic acids and proteins in yeast protoplasts has been directly proved biochemically (12, 13, 43, 57).

Protoplasts of *S. cerevisiae*, when cultivated in liquid nutrient media, form only aberrant cell walls consisting of a fibrillar network (47, 71). This network may be considered analogous to the glucan fibrils representing one of the structural components of normal yeast cell walls. The fibrillar network formed around the protoplasts is not able to induce normal morphogenesis, and the protoplasts do not revert to normal cells in liquid media.

I found (62, 63) a very low rate of regeneration (about 0.1%) of protoplasts prepared by autolysis of the cell wall when cultivating them on the surface of solid agar media. As has already been mentioned, a small number of spheroplasts may be present in the protoplast population, and it is possible that the regeneration observed on the surface of solid media is in fact a reversion of these spheroplasts. They may arise more frequently when preparing protoplasts by the wall autolysis procedure.

The quantitative reversion of *S. cerevisiae* protoplasts takes place after embedding them in firm gel media. The quantitative reversion of protoplasts prepared by autolysis was first described in 1961 (69) (using the procedure of embedding in gelatin gels) and later confirmed by Svoboda (104) for protoplasts prepared with snail enzymes. Svoboda (101) obtained the same results when embedding protoplasts in agar gels. Under these conditions, both wall components (i.e., fibrillar network and amorphous matrix) are synthesized synchronously, and complete cell walls regenerate. The fact that the same effect on wall regeneration is displayed by proteineous gels (gelatin) as well as by polysaccharide gels (agar) indicates that physical factors play a decisive role in this respect.

The fact that in liquid media the formation of fibrils is uncoupled from that of the matrix offers a unique opportunity for studying the biosynthesis of fibrils separately from the synthesis of matrix, which is not possible during normal cell-wall reproduction.

Synthesis of the Fibrillar Component

The structural element of the fibrillar wall component synthesized on the surface of *S. cerevisiae* protoplasts in liquid media is a microfibril of polysaccharide nature. Microfibrils are aggregated to

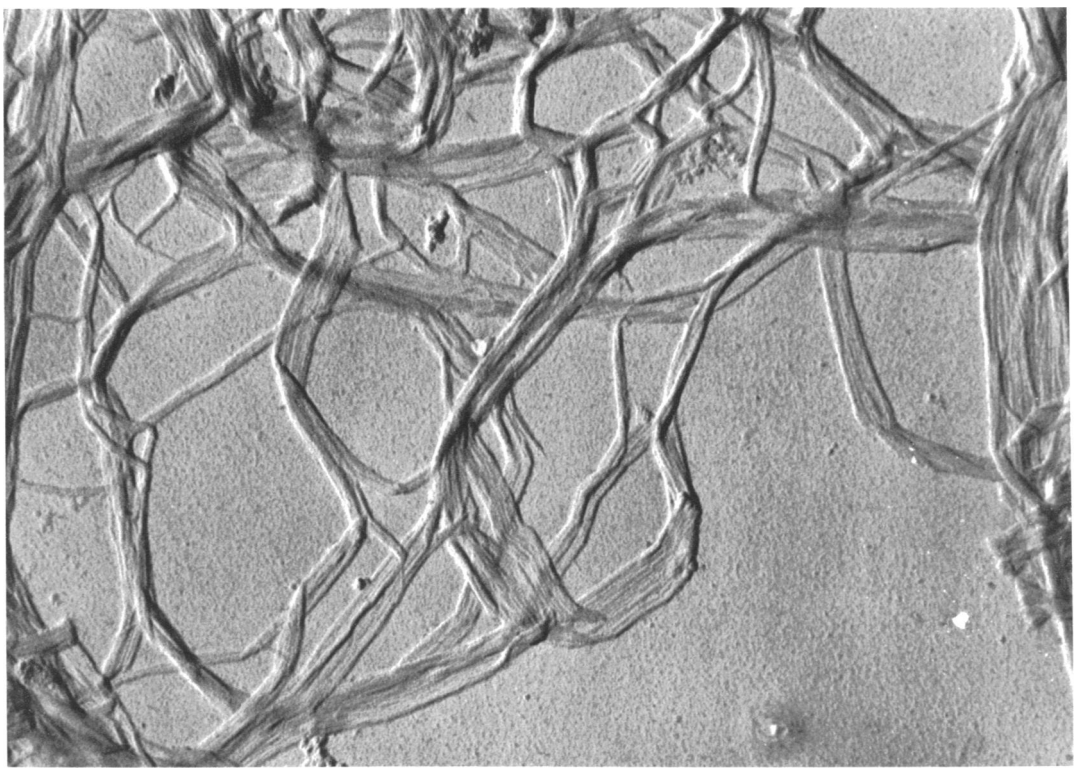

FIG. 2. *Part of the fibrillar network formed on the surface of a Saccharomyces cerevisiae protoplast incubated in liquid medium for 2 hr. Microfibrils aggregate to thicker bundles which are irregularly intermeshed. Shadowed specimen.* × *30,000 (Courtesy of Marie Kopecká.)*

thicker bundles which are intermeshed, forming a fibrillar network around the protoplast (Fig. 2).

Chemical composition and structure of microfibrils. The fibrillar network can easily be isolated by centrifugation after osmotic lysis of protoplasts. The purification of the fraction, however, is extremely difficult. In particular, the detached remnants of incompletely digested cell walls and bud scars may cause misleading errors in evaluating the results of chemical analyses. This must be taken into consideration when evaluating all the chemical analyses that have been made to date.

Eddy and Williamson (15) analyzed the fibrils (from aberrant cell walls) in *S. carlsbergensis* protoplasts cultivated in liquid media. They found mannose, glucose, a relatively high content of *N*-acetyl-glucosamine (27 to 33% dry weight), and traces of amino acids. Results similar to those for the high content of glucosamine were obtained by Garcia-Mendoza and Novaes-Ledieu (26) in regenerating cell walls of protoplasts of *C. utilis*. These investigators concluded that this could be related to chitin. Recently, D. R. Kreger and M. Kopecká (*unpublished data*) proved chitin in X-ray diagrams of isolated fibrillar networks synthesized by *S. cerevisiae* protoplasts. Bacon et al. (3), however, have shown that cell wall residues in yeast protoplast preparations contain bud scars which are composed mainly of chitin. Thus, the high content of glucosamine in chemically analyzed fractions of fibrils may be due to contamination with bud scars. The microfibrils are resistant to chitinase action (M. Kopecká, *unpublished data*), which supports this view.

The most valuable data concerning the polysaccharide composition of microfibrils were published by Eddy and Woodhead (16). They isolated a glucan fraction from *S. carlsbergensis* protoplasts incubated in liquid media and estimated the molecular weight of this glucan to be $1.1 \times 10^5 \pm 0.3 \times 10^5$. The microfibrils from incubated *S. cerevisiae* protoplasts have recently been analyzed by D. R. Kreger and M. Kopecká (*unpublished data*), using X-ray analysis; the X-ray diffraction diagrams corresponded fully to paramylon and almost completely to hydroglucan. This means that the main component of microfibrils is highly crystalline $\beta(1 \rightarrow 3)$ glucan. No other evidence is available about the structure of

this glucan, especially about the presence of β(1 → 6) links. Thus, it remains uncertain how the glucan of microfibrils corresponds in structure to the analogous glucan from normal yeast cell walls.

The microfibrils from protoplasts of *S. cerevisiae* easily dissolve in alkali, but they are very resistant to acid hydrolysis (47). They disappear only when boiled with 2 N hydrochloridic acid for 6 hr (49). The fibrils disappear after 24 hr when treated with the complex of polysaccharide-splitting enzymes in snail gut juice.

The thickness of microfibrils in isolated and metal-shadowed preparations was estimated to be 20 nm for *S. cerevisiae* (47, 71). Negative staining, however, revealed much thinner elements of 6 nm in diameter for *S. carlsbergensis* (A. A. Eddy, *unpublished data*) and for *S. cerevisiae* (49). The length of microfibrils cannot be determined in purified preparations because shorter fragments may arise artificially by fracturing of fibrils. No transverse periodicity has been found in native material. After incomplete hydrolysis with acids, transverse periodicity of about 50 and 100 nm, respectively, is revealed (49).

No evidence is available about the molecular organization of the microfibrils.

Fibrillar network. The formation of microfibrils in liquid media starts after about a 20-min lag in protoplasts prepared by snail enzymes (47) and after a 2-hr lag in protoplasts prepared by autolysis of the cell wall (71). Microfibrils aggregate to thicker bundles which are randomly intermeshed. The number of microfibrils arranged into bundles differs considerably, depending on the conditions of cultivation, indicating that this aggregation is influenced by physical forces (47). The distance between the longitudinal axes of microfibrils joined to bundles was estimated in frozen-etched material as 12 nm. Assuming a 6-nm thickness of microfibrils, a 6-nm interspace results between the microfibrils (47). The texture of bundles in the isolated network is completely irregular. This arrangement is not artificial, as the same picture has been observed in replicas of frozen-etched protoplasts (75, 80). In the fibrilar network isolated from *S. cerevisiae* protoplasts, an area can usually be found in which the density of the bundles is higher than elsewhere and from which the bundles seem to radiate in all directions (47, 49).

During the incubation of protoplasts, the density of fibrils increases, 6-hr-old protoplasts having a dense network consisting of broad bundles of microfibrils. In this stage, the network can also be observed in situ, as it shows weak fluorescence when primulin is used as a fluorochrome (99). More-detailed evidence concerning the rate of synthesis has been obtained by the use of ^{3}H-glucose (75, 83). The radioactivity of the fractions of fibrils in the course of cultivation is demonstrated in Fig. 3. It appears that the label started to increase after a 20-min lag. The incorporation of ^{3}H-glucose into fibrils was repressed after 3 to 6 hr of incubation, whereas the incorporation into other protoplast components continued.

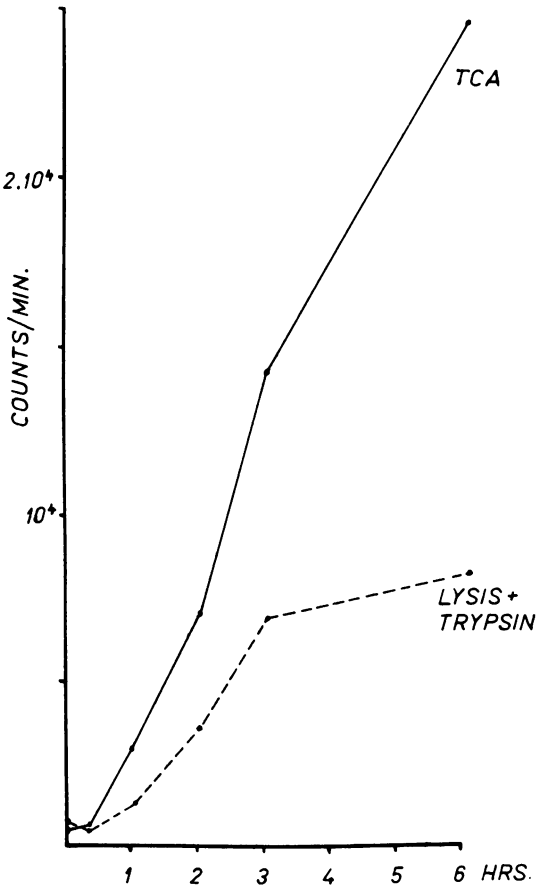

FIG. 3. *Incorporation of ^{3}H-glucose into protoplasts at intervals of incubation with 100 mCi of ^{3}H-glucose per mmole. Total incorporation (solid line) measured as trichloroacetic acid precipitate. Incorporation of ^{3}H-glucose into fibrils (broken line) estimated after osmotic lysis of protoplasts and subsequent treatment with trypsin.*

High-resolution autoradiography has demonstrated that the wall remnants and bud scars do not incorporate ^{3}H-glucose; neither are they centers inducing the formation of fibrils. Similarly, the spider-like formations described above do not preferentially incorporate labeled glucose (83).

There are two types of protoplast growth: iso-

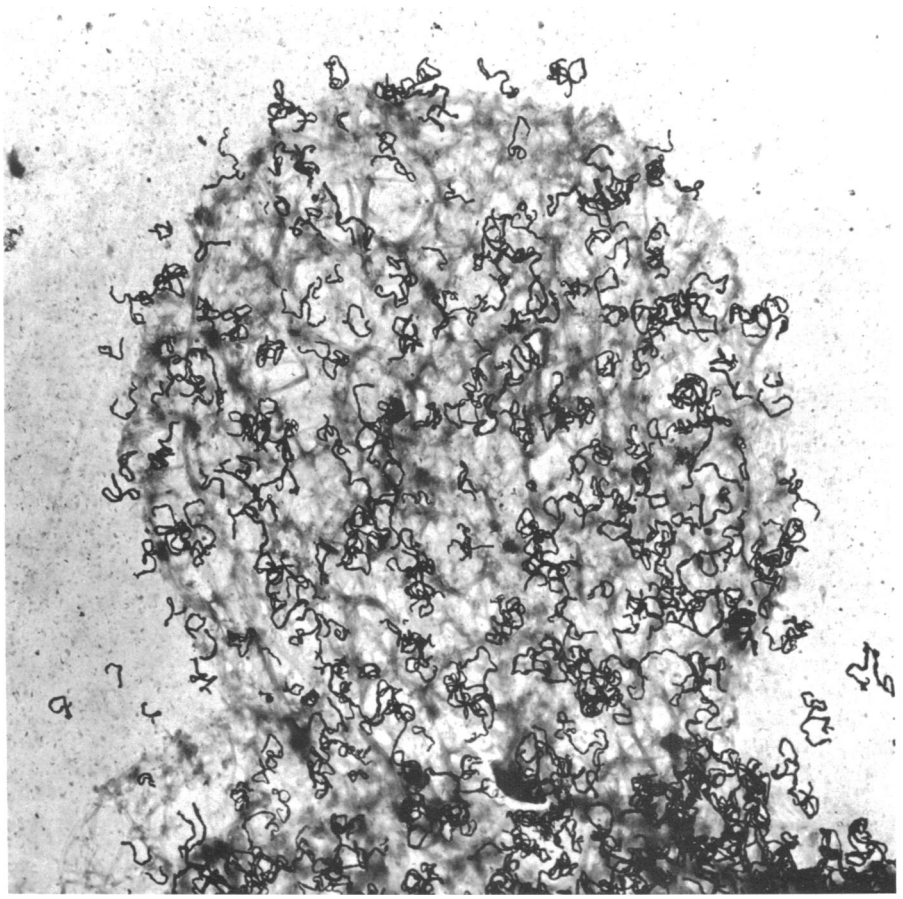

FIG. 4. *High-resolution autoradiography of the fibrillar network formed on the surface of a Saccharomyces cerevisiae protoplast. Incubation of protoplasts in synthetic nutrient medium containing 100 mCi of glucose-6-^{3}H per mmole for 6 hr. Grains are uniformly distributed.* × *6,000.*

diametric, occurring in liquid media, and polar, observed on the surface of solid media and occasionally in liquid media. High-resolution autoradiography of protoplasts incubated in liquid media after pulse labeling and subsequent chases have shown that the fibrils are synthesized isotropically around the whole surface of the protoplast (Fig. 4) and that the fibrillar network grows by interposition of new microfibrils (75, 83). The polar growth of protoplasts on the surface of solid media is based on the formation of herniae, as demonstrated by time-lapse microcinematography (*unpublished data*). The surface of the hernia is naked, but soon new bundles of microfibrils appear (49). If the process of herniation repeats several times a tubular network arises, revealing a different density of fibrils, according to the age of subsequent herniae.

Synthesis of glucan macromolecules. The biochemistry of glucan synthesis has not been studied in yeast protoplasts, but it can be presumed that the pathways are similar (or identical) to those in normal yeast cells. Uridine diphosphate (UDP)-glucose is known as a main sugar nucleotide presursor of yeast glucan (7).

Little is known about the cellular localization of the synthesis of glucan molecules. Rost and Venner (91) proved by chromatography the presence of UDP-glucose in the medium of incubated *S. fragilis* protoplasts and suggested that it may represent a precursor for the extracellular synthesis of cell wall components. Moor and Mühlethaler (59) proposed that the particles attached to the outer surface of the plasmalemma of normal yeast cells could be the actual site of glucan synthesis.

The addition of proteolytic enzymes, such as trypsin and Pronase, to the cultivation medium does not affect the synthesis of fibrils (49). This fact indicates that the synthesis of wall glucan

does not take place either extracellularly or on the surface of the plasma membrane. One can suggest that the glucan macromolecules, representing the building blocks of glucan microfibrils, are formed inside the protoplasts and then released to the outside. The mechanism of their transport across the plasma membrane remains unknown.

Inhibition of microfibril formation. The formation of microfibrils in protoplasts incubated in a nutrient medium can be completely inhibited by the addition of snail enzymes. This inhibition is reversible; after removal of the snail enzymes a normal fibrillar network forms around the protoplasts (105). The mechanism of this inhibitory effect is not known. The snail enzymes may degrade the glucan macromolecules before their organization in the supramolecular complex of the microfibrils or interfere directly with this organization. However, it must be emphasized that the fibrils, once formed, are extremely resistant to snail enzymes. Treatment of the isolated network with snail enzymes for only 24 hr will dissolve it (M. Kopecká, *unpublished data*).

Ribonuclease entirely inhibits the formation of fibrils (48) at a concentration which does not lyse the protoplasts (67). Ribonuclease was added to the protoplasts which were osmotically stabilized with 0.6 M mannitol. Then the ribonuclease was replaced by liquid nutrient medium. The protoplasts remained alive, but they did not show any signs of growth and no fibrils were formed. As shown by Schlenk and Dainko (92), ribonuclease impairs the function of the plasma membrane. The inhibitory effect of ribonuclease on growth and microfibril formation can thus be explained by the irreversible damage of the protoplast plasma membrane.

No fibrils are synthesized if glucose in the nutrient medium is replaced by 2-deoxyglucose. An incomplete inhibition takes place in media in which the glucose-to-deoxyglucose ratio is 4:1, and deoxyglucose is not incorporated into the fibrils (19, 106). Because deoxyglucose interferes with the synthesis of polysaccharides (20, 46), it may be concluded that the formation of microfibrils is directly dependent on the effective synthesis of glucan molecules.

The effect of cycloheximide (actidione) on cell wall synthesis in protoplasts of *S. cerevisiae* is interesting. The formation of a fibrillar network was not affected by cycloheximide in liquid media (82), whereas growth of protoplasts and protein synthesis were inhibited immediately (96). Fibrils formed even when the yeast cells were treated with cycloheximide 2 to 4 hr before their conversion to protoplasts. Hence, it might be presumed that in freshly prepared yeast protoplasts all structural precursors of fibrils and the respective polymerizing enzymes are already at hand. Thus, either the construction of extracellular fibrils is independent of protein synthesis or, more probably (because of the inhibitory effect of deoxyglucose), the synthesis of glucan is relatively independent of protein synthesis. The synthesis of the wall matrix, however, is inhibited immediately by cycloheximide, as will be shown later.

Fluorouracil has no effect on the formation of fibrils in *S. cerevisiae* protoplasts (95).

Site of microfibril formation. Moor and Mühlethaler (59), using the freeze-etching technique, found the outer face of the plasma membrane of yeast cells covered with globular particles packed into a regular hexagonal arrangement in some areas. They suggested that the particles represented structural components of the plasma membrane and that the individual particles were a site for the synthesis of amorphous cell wall glucan, whereas those concentrated in the hexagonal patterns synthesized the crystalline glucan fibrils. Later on, Matile et al. (55) succeeded in isolating the particles and characterizing them as mannan-protein, and they suggested that the particles represent building blocks to be incorporated into the fibrillar network of the cell walls.

Particles of a diameter of about 10 nm, which may be considered identical to those found by Moor and Mühlethaler (59), also cover the outer surface of the plasma membrane of protoplasts (79, 97). They are firmly bound to the plasma membrane (Fig. 5), and only the particles along the invaginations are always detached during fracturing. Surprisingly, the treatment of freshly prepared protoplasts with proteinases (trypsin and Pronase) did not cause structural changes of the particles (75, 80). The same results were obtained by using snail enzymes and succesive treatment with Pronase and snail enzymes. Similarly, the treatment of protoplasts with actidione and the same treatment of yeast cells before their conversion to protoplasts did not influence the particles. Thus, it seems improbable that the particles are composed of proteins or glycoproteins. This resistance of particles to enzyme action was also tested physiologically. When the treated protoplasts were washed free of the respective enzymes and incubated in liquid nutrient medium, the formation of fibrils occurred as in the untreated protoplasts (A. Svoboda, *unpublished data*). However, these results would be evaluated quite differently if the particles are located *inside* the unit membrane; their resistance to the enzymes used would be easy to understand.

Freeze-etching studies of protoplasts in the process of synthesizing microfibrils have shown that the fibrils are always separated from the par-

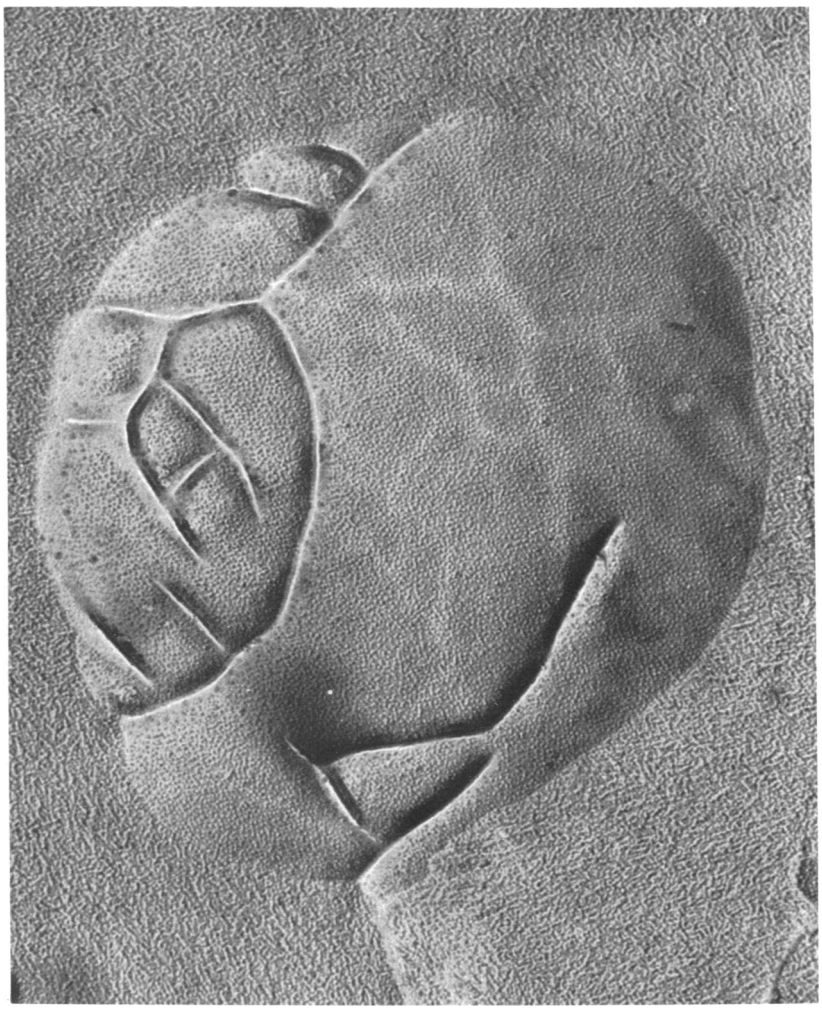

FIG. 5. *Frozen-etched yeast protoplast (Saccharomyces cerevisiae) in which the outer face of the plasma membrane was exposed by fracturing. The protoplast surface is covered with granules measuring about 10 nm in diameter. The surface reveals deep and elongated invaginations. No profile or ridge which could be ascribed to any other layer can be found at the junction of the protoplast and the surrounding frozen medium. (The background eutecticum reveals a very fine structure.)* × *40,000.*

ticles by a thin layer of nonetchable material (75, 80; Fig. 6). This nonetchable layer may be identical to the artificial eutectic layer which arises during the freezing of protoplasts (79) or to the outer leaflet of the split-unit membrane. In any case, no direct structural connection was found between the microfibrils and the particles of the plasma membrane.

These findings indicate, contrary to the view of Moor and Mühlethaler (59) and that of Matile et al. (55), that the plasma membrane granules are not directly involved in the synthesis of the fibrillar wall framework and that the microfibrils are formed free from any structural connection with the plasma membrane.

This statement makes the elucidation of the mechanism of formation of the microfibrils very difficult. One possible explanation is indicated by the experiments of Eddy and Woodhead (16), who obtained in vitro reaggregation of fibrillar elements from dissolved glucan. One can suggest, according to this finding, that the protoplasts secrete barely soluble glucan or its supramolecular aggregates in the form of hypothetical elementary fibrils which form a concentration gradient around the protoplasts. The microfibrils could then originate through self assembly (crystallization) of these accumulated elements. This concept is in accord with the fact that proteolytic enzymes do not inhibit the formation of microfibrils; more-

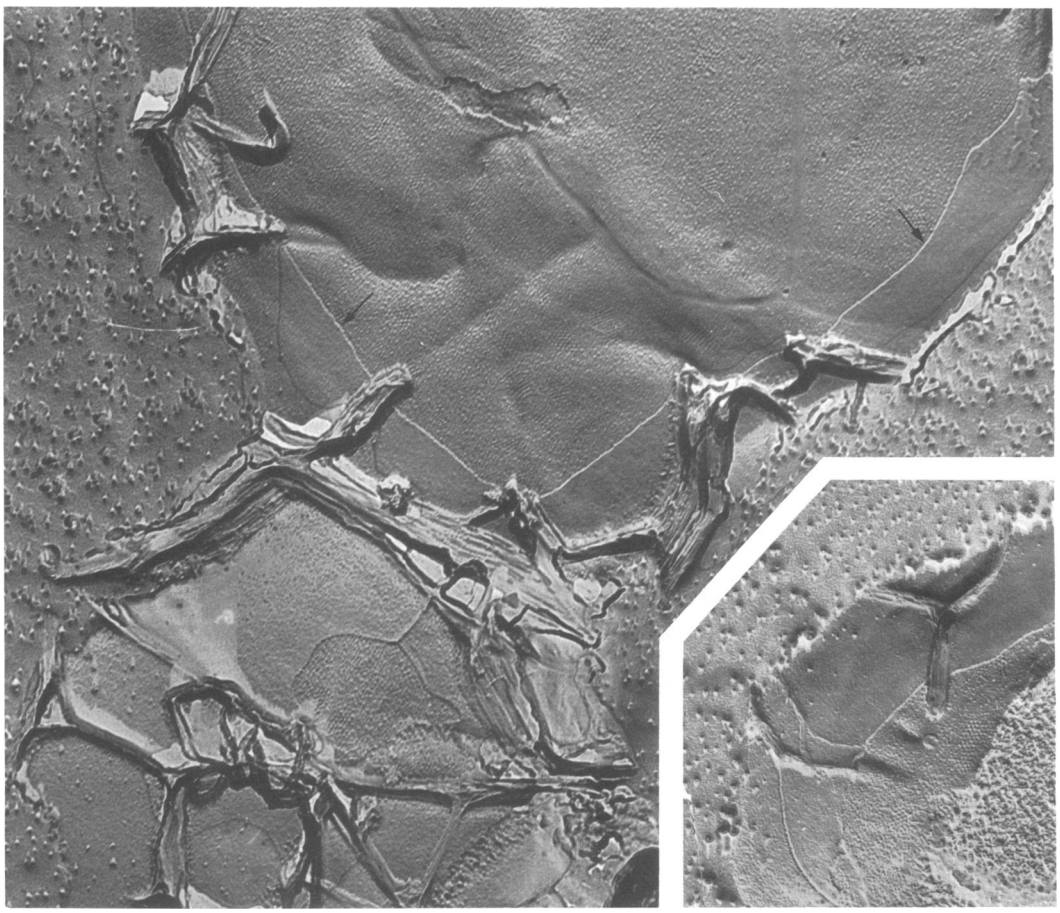

FIG. 6. *Frozen-etched Saccharomyces cerevisiae protoplast incubated in liquid medium. The fibrillar network, where it has not been removed by cleavage, is separated from the outer face of the plasma membrane covered with particles by a non-etchable eutectic layer (arrows). This artefact shows clearly (see also inset) where the ice has sublimed.* × *38,000.*

over, it conforms with the explanation of the effect of gelled media on the regeneration of complete cell wall, as will be described later.

Conclusion. A structural unit of the fibrillar wall component synthesized by *S. cerevisiae* protoplasts in liquid media is a microfibril 6 nm in diameter and of indefinite length. It is composed of crystalline glucan. Secondarily, the microfibrils aggregate to thicker bundles which are irregularly intermeshed, forming a fibrillar network around the protoplasts. The microfibrils are formed independently of preexisting structure, such as wall remnants or bud scars. Their formation in *S. cerevisiae* protoplasts is repressed after about 6 hr of incubation. The formation of microfibrils is independent of protein synthesis but is sensitive to inhibition of polysaccharide synthesis. The direct participation of plasma membrane structures in the formation of fibrils has not been proved. The mechanism of microfibril construction is not clear. One can speculate that the microfibrils arise through extracellular crystallization from secreted glucan macromolecules or larger units which form a concentration gradient around the protoplasts.

Synthesis of the Amorphous Component (Matrix)

The amorphous component of the yeast cell wall consists mainly of polysaccharides (mannan and amorphous glucan), proteins, and lipids. The molecular architecture of the wall matrix has not been definitely solved, although many proposals have been made [reviewed by Phaff (87), Northcote (85), Nickerson (84) and Lampen (51)]. Little is known about the synthesis of wall proteins (94) and nothing about that of lipids. Contrary to this, there is some evidence available concerning the origin of mannans.

Synthesis and secretion of mannans. Yeast protoplasts can continue in the synthesis and secretion of mannan and of mannanprotein enzymes, such as invertase, melibiase, acid phosphatase, etc. [*see* review by Lampen (51)]. The biosynthesis of wall mannan from guanosine diphosphate ^{14}C-mannose has been studied by Behrens and Cabib (4) in a cell-free system consisting of disrupted yeast protoplasts and was found to be associated with the particulate fraction. A lipophilic mannosyl intermediate is suggested to be the immediate precursor for mannan biosynthesis (108). The site of mannan synthesis remains open. According to one hypothesis (1, 50), mannan is manufactured in close association with the plasma membrane. Other hypotheses assume the excretion of polysaccharides by reverse pinocytosis of vesicles derived from the Golgi apparatus. In *S. cerevisiae* protoplasts, the direct observation either on ultrathin sections (77) or on freeze-etching replicas (79, 80) did not prove, however, any morphological signs of reverse pinocytosis.

Thus it appears that the protoplasts of *S. cerevisiae*, when cultivated in liquid media, synthesize and secrete wall mannan and probably all other constituents of the wall matrix, although the complete cell wall is not formed. In liquid media there do not exist appropriate conditions for the organization of the matrix architecture, and these wall polymers, unlike the precursors for glucan fibrils, are lost in the medium. Only the embedding of protoplasts in gelled media provides suitable physical conditions for the construction of the wall matrix.

Formation of matrix in gel media. In protoplasts embedded in media containing 30% gelatine or 2% agar, a thin layer of the cell wall material can be detected outside the plasma membrane after the first hour of incubation (Fig. 7). These layers can be isolated easily after osmotic lysis of the protoplasts. They consist of a fine network of fibrils incompletely masked by an amorphous matrix (71). In ultrathin sections, they appear as electron-transparent layers, their thickness being about 15 nm. No analogical membranes can be detected, either after lysis or in ultrathin sections, in freshly prepared or in briefly incubated protoplasts.

In the course of further incubation, the membranes become thicker (Fig. 8), and after about 15 hr, their thickness reaches 200 to 300 nm [i.e., thicker than the cell wall of normal cells (77, 78)]. The ultrastructure of regenerated cell walls differs slightly from that of normal cells. The electron-dense outer layer is found considerably thickened and discontinuous. The regenerated walls usually

FIG. 7. *First steps of cell wall regeneration in a Saccharomyces cerevisiae protoplast (2 hr after embedding in gelatine medium) showing a thin complete wall. Protoplasts were resuspended and osmotically disrupted, and the membranes of wall material were isolated by centrifugation and shadowed for electron microscopy.* × *6,000.*

display a distinct lamellation on ultrathin sections when compared with normal cells. Both the outer and inner faces of regenerated walls are not smooth but reveal incompletely masked fibrils, as demonstrated after negative staining and in frozen-etched replicas. Very frequently, remarkable structural abberations can be found in the forms of local thickening, deep invaginations of wall material into the cytoplasm, doubling of large areas of the wall, and dystopic translocations of wall material to the interior of the cytoplasm (78).

The protoplasts enveloped with regenerated walls revert to normal cells by budding (63, 69, 77, 78; Fig. 9).

The formation of matrix in gelatin media is a function of the concentration at a given temperature or of the temperature at a given concentration of gelatin (69, 70, 76). In lower gelatin concentrations, the fibrils are synthesized normally, whereas the formation of the matrix is strongly reduced. Such protoplasts do not revert to normal cells. Thus, it appears that the rate of matrix formation is a function of certain physical properties of the gel medium surrounding the protoplasts. Two facts exclude the chemical influence of gelatin. (i) Hydrolyzed gelatin does not provoke wall regeneration (70). (ii) The same positive effect as gelatin is displayed by polysaccharide gels [e.g.,

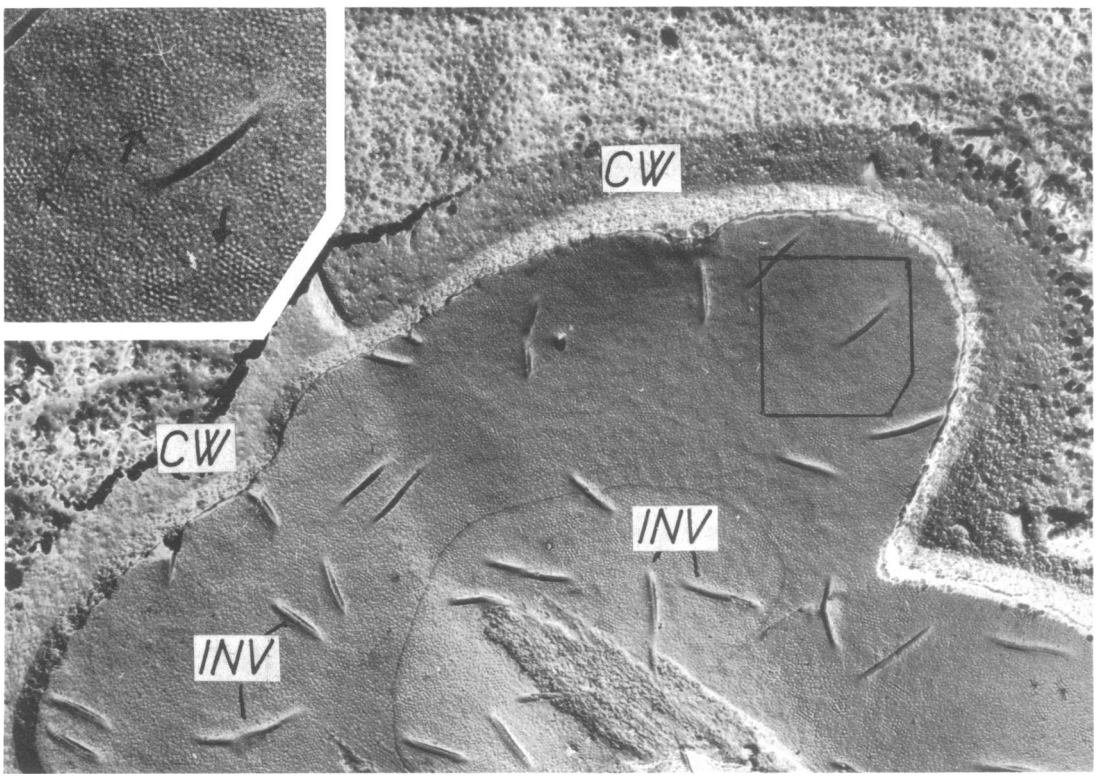

FIG. 8. *Freeze-etching of a protoplast (Saccharomyces cerevisiae) regenerating the cell wall after embedding into high-percentage gelatine. The cross-fractured cell wall (CW) reveals rough structures. The plasma membrane is covered with particles showing hexagonal arrangement (arrows in inset taken from area shown) in some places. The form of the invaginations (INV) corresponds to that of normal cells rather than that of Fig. 5.* × *26,000.*

agar (101)]. Gels probably prevent the diffusion or loss into the medium of the precursors of the matrix and of polymerizing enzymes, respectively.

This view is strongly supported by experiments in which the physical conditions were changed in the course of protoplast cultivation (70, 76, 105). If protoplasts are preincubated in high-percentage gelatin and transferred to liquid media within about 4 hr, they never revert, and only a fibrillar network can be demonstrated on their surface. If they are transferred later, complete walls regenerate even in liquid media and reversion takes place. The same results are obtained if the gelatin gel embedding the protoplasts is liquefied for the appropriate periods by raising the temperature. At that time (after the preincubation of protoplasts in gelled media lasting about 4 hr), the protoplasts are already enveloped by a thin coherent layer of the regenerating wall which evidently can take over the role of the gel medium (or of the normal cell wall).

Inhibition of matrix formation. The formation of the matrix can be inhibited by enzymes, antibiotics, and deoxyglucose.

The addition of snail enzymes to gelatin media completely inhibits the formation of both fibrils and matrix, whereas the protoplasts continue to grow. This inhibition of wall synthesis is reversible at any time. These protoplasts, when resuspended and reembedded in the gelatin medium without snail enzymes, synthesize a new, normal cell wall (105). The reversion of walled protoplasts depends, however, on the period during which they are exposed to the snail enzymes. These relations will be discussed later.

Cycloheximide (actidione) added to the gelatin inhibits completely the formation of the matrix, and the formation of the fibrillar network remains unaffected (82). When the yeast cells are incubated in the presence of cycloheximide 2 to 4 hr before their conversion to protoplasts and the latter are then embedded in gelatin containing cycloheximide, the results are the same; i.e., only fibrils without matrix are found (82). This is in good agreement with the effect of actidione on the synthesis of wall components in normal cells. Sentandreu and Northcote (94) found that actidione inhibits the incorporation of labeled threo-

nine into the wall but only partially inhibits the incorporation of labeled glucose. Similarly, Elorza and Sentandreu (18) found a reduction in the synthesis of mannan from 41.5 to 6.9% after actidione treatment but an increased synthesis of glucan (from 58.5 to 93%). From these experiments it appears that the formation of the wall matrix, contrary to the formation of fibrils, is in close dependence on protein synthesis. It has been found (20) that actidione does not interfere with the synthesis of mannans in a cell-free system. Thus it may be concluded that only synthesis of proteins is inhibited by actidione. The coincident effect on the secretion of mannans could be explained by the hypothesis that the mannan molecules can be transported only as a complex with proteins.

The synthesis of the matrix is more sensitive to the presence of 2-deoxyglucose than that of the fibrils. If 2-deoxyglucose replaces 5% of the glucose in the gelatin medium, the surface structures formed are of a fibrillar nature with a reduced amount of the matrix substance (19, 106). When the embedding medium contains only 2-deoxyglucose, the growth of protoplasts, as well as cell wall regeneration, is completely inhibited. This inhibition is competitive and can be abolished by adding excess glucose to the medium. It is possible to find a concentration of deoxyglucose not inhibiting growth of the protoplast but inhibiting the synthesis and secretion of mannan-protein (20),

Conclusion. Protoplasts of *S. cerevisiae* probably synthesize and release all structural components of the wall matrix. Their assembly into the wall structure requires the creation of special physical conditions in the protoplast environment (embedding into gelatin or agar gel media), permitting the extracellular accumulation of matrix precursors. The formation of the matrix is, unlike that of the fibrillar skeleton, directly dependent on protein synthesis and more sensitive to deoxyglucose than is the formation of fibrils.

CELL WALL REGENERATION IN PROTOPLASTS OF OTHER YEAST GENERA

Budding Yeast

In the protoplasts of all yeast species that reproduce by budding, the regeneration of the cell wall proceeds principally in the same way as described for *S. cerevisiae*. On the surface of protoplasts cultivated in liquid media, only a fibrillar network forms and no mass reversion to normal cells takes place. Regeneration of the cell wall and quantitative reversion occur only after embedding the protoplasts in gel media. The reversion of protoplasts has been described for *S. carlsbergensis* (42, 103), *S. fragilis* (90, 91), *S. willianus* (90), *C. utilis* (102), and *C. albicans* (11).

Uruburu et al. (112) described osmotic-resistant tubular forms in growing *C utilis* and *Pichia polymorpha* protoplasts. Their aberrant cell walls consisted of a fibrillar framework. No data for the reversion of these tubular forms to normal cells have been reported. Garcia-Mendoza and Novaes-Ledieu (26) found a high concentration of chitin (22 to 27%) in these fibrous abberant walls and suggested that mannan present in normal cell walls is substituted by chitin in cell wall regenerating protoplasts. It remains questionable, however, whether this high content of glucosamine is not due to the contamination with bud scars originating from normal cell walls.

Fission Yeast

The synthesis of the complete cell wall by protoplasts of *Schizosaccharomyces pombe* is in contrast to the budding yeasts, attainable in liquid media (102). In liquid media the protoplasts of *Schizosaccharomyces* start to form a fibrillar network which is very similar to that of *Saccharomyces cerevisiae*. The formation of fibrils, however, is not repressed after 6 hr as in *Saccharomyces*, whereas in the course of further incubation the number of fibrils steadily increases; thus, the zone of densely intermeshed fibrils around the protoplasts may be as much as 1 μm in depth (81). After about 15 hr, the protoplasts become osmotically stable. At this interval, deposits of an amorphous substance can be detected among the fibrils close to the plasma membrane. Thereafter the regeneration of new cells occurs.

The relation between the ability of *Schizosaccharomyces* protoplasts to regenerate the cell wall even in liquid media and the chemical composition of normal cell walls is not yet clear. Whether the cell walls of *Schizosaccharomyces pombe* contain mannans is uncertain. They contain, however, galactomannans (30*a*) and $\alpha(1 \rightarrow 3)$ linked glucan (2*a*). How the specific properties of these polymers could influence the cell wall formation in liquid media is not known. It may be suggested that the unrepressed synthesis of fibrils is the reason the protoplasts of fission yeast can revert also in liquid media. A very broad and dense basket of fibrils enveloping the protoplasts may act in the same way as gel media; i.e., to prevent the release of structural components of the matrix.

The protoplasts of *S. pombe* were often found connected with remnants of the original wall (32, 81, 89). Thus, it might be speculated that the wall remnants act as an acceptor for binding the polymers of reconstituting walls and that this

facilitates wall regeneration in liquid media. Quantitative experiments (107), however, have shown that *S. pombe* protoplasts regenerate in a higher percentage than would correspond to the number of protoplasts with attached wall remnants. Therefore, even in fission yeast the wall remnants cannot be considered a necessary primer for wall regeneration.

In *Schizosaccharomyces* protoplasts embedded in gel media, the synthesis of fibrillar groundwork and matrix takes place synchronously (33, 90). New cell walls are already formed within 8 hr, differing in ultrastructure from normal cell walls. At this stage numerous instances of binary fission of protoplasts are seen, the septum probably being formed by centripetal growth. Cell walls of typical structure are formed only in the cells of the next generation.

Havelková and Menšík (37) found a great number of Golgi bodies in regenerating *S. pombe* protoplasts. They obtained evidence that the development of dictyosomes was closely related to the synthesis of some of the cell wall components, probably of the matrix.

Other Yeasts

Reversion of *Nadsonia elongata* protoplasts, as well as the regeneration of the cell wall, was studied by Svoboda (102) and Havelková (34, 39). It was found that *N. elongata* protoplasts are able to regenerate the cell wall also in liquid media in a manner similar to *S. pombe*. A dense, fibrillar network forms first, followed by the matrix in about 70% of the protoplasts. In gelled media, both components are organized simultaneously, the protoplasts starting to divide after about 20 hr. The outer electron-dense layer present in normal cell walls is missing in regenerated walls, which become completely normal only during the next generation. No nuclear division occurs in the protoplasts until the new cell wall appears.

Protoplasts of *Lipomyces lipofer* were studied by Havelková (35, 38). Those grown in gelled media regenerated a new cell wall within 30 hr, including the slime capsule, but only under aerobic conditions (in very thin layers of gelatin media). Under semianaerobic conditions (e.g., in the depth of the gelatin medium), only fibrils originated and the protoplasts gradually died. *L. lipofer* cells are strict aerobes, and it appears that the biosynthesis of the fibrillar component is less sensitive to lack of oxygen than the biosynthesis of the wall matrix. In liquid media, the rate of regeneration is rather limited (only about 10%), and most protoplasts grow into giant forms having only a fibrillar network on their surface. They may survive several weeks without reversion to normal cells.

Reversion of the protoplasts of the dimorphic yeast *Endomycopsis fibuliger* was studied by Svoboda and Máša (103). Both forms, yeastlike and filamentous, produce protoplasts capable of regeneration in gelatin as well as in liquid media. The type of regenerated cells depends only on the cultivation conditions, without any relation to the type from which the protoplasts were derived. The protoplasts obtained from the filamentous type revert as yeastlike cells under anaerobic conditions and those from yeastlike cells as filaments under aerobic conditions. Dimorphism in yeasts and moulds is induced by environmental factors probably through influencing the chemical composition and the structure of the cell wall. The experiments by Svoboda and Máša clearly show that the existing cell differentiation in one or another dimorphic type can be completely abolished by conversion to protoplasts.

The reversion of protoplasts, presumed to involve wall regeneration, has also been described for *Endomyces magnusii* protoplasts grown in liquid media (56). No detailed data on the wall biosynthesis have been published.

Conclusion

Protoplasts of most budding yeasts form only a fibrillar wall component when cultivated in liquid media, and never revert. Regeneration of a complete cell wall and subsequent reversion occur only after embedding protoplasts in gelled media. The protoplasts of all other yeasts studied can regenerate a cell wall even in liquid media. In these media, the construction of the two wall components, fibrillar and amorphous, is not synchronized. Protoplasts first form a dense fibrillar network, whereupon the amorphous component is deposited.

ROLE OF THE CELL WALL IN CELLULAR MORPHOGENESIS

A plant cell without a cell wall is definitely limited in its existence. (There is only one exception: stable bacterial L-forms of a protoplast type which will be mentioned later.) The same fate awaits protoplasts forming abberant cell walls that consist only of a fibrillar skeleton, although its existence can considerably influence the morphology of protoplast growth. Only protoplasts that are able to synthesize a complete cell wall can divide and thus revert to the cell type of the original strain. These facts offer excellent opportunity for some statements about not only the control of wall biosynthesis but also the biological role of the cell wall, especially in the control of morphogenetic processes.

Fate of Protoplasts Without Cell Wall

Protoplasts capable of synthesizing a new cell wall revert to normal reproducing cells. Thus, the interesting question may arise whether the protoplasts not regenerating the cell wall, or forming only aberrant walls (fibrillar network), can remain alive for a longer time or even propagate indefinitely in a protoplast state. Little experimental evidence has been furnished, however, in this respect.

The protoplasts cultivated in liquid media, depending on the species, either regenerate the cell wall and revert or undergo metabolic lysis (44). The protoplasts embedded in gelled media always revert and, under these conditions, a long-term selective blocking of wall synthesis is difficult to achieve. It appears that the only experimental possibility is to cultivate protoplasts on the surface of solid media (66) where metabolic lysis does not occur. *Saccharomyces cerevisiae* protoplasts grow abundantly on the surface of agar media. After about 48 hr of normal growth, the structure of protoplasts undergoes considerable change. The vacuoles disappear, the viscosity of the cytoplasm evidently increases as well as its refractive index, and the protoplasts, having originally a varied shape, become round. They are multinuclear as their growth is accompanied by nuclear division. These dense spherical formations slowly go on growing, and their number increases by forming herniae which split off from the mother protoplasts. Only a fibrillar network is demonstrable on their surface. This development usually lasts more than five days, and later most of the protoplasts gradually disintegrate. In some cases, however, the development continues for several months, and microcolonies arise that consist of protoplasts of different size at the periphery and of debris of degenerated protoplasts in the center. However, the cultivation conditions cannot be defined, making the described observations not readily reproducible. In any case, it seems that the yeast cell can live and multiply in a protoplast state for a very long period under appropriate conditions.

The limited life of protoplasts without walls has also been reported in other plant cells such as moulds [reviewed by Strunk (100)], higher plant cells [reviewed by Cocking (9)], and green algae (24). It is common knowledge that bacterial spheroplasts and unstable L-forms easily revert to original cells. The protoplasts of bacteria were generally believed to have lost their regenerative capacity (6), and only recently did Landmann and his co-workers (52, 53) report the reversion of *Bacillus subtilis* protoplasts after embedding them in gelled media. The majority of stable L-forms of bacteria reproduce as such, having aberrant walls on their surface. Only stable L-forms of the protoplast type can live and even multiply in the naked state (54, 93, 110, 111); no trace of wall material or structure is found on their surface other than the plasma membrane (54, 109). This type of stable L-form represents a unique case —a cell can reproduce indefinitely without having a cell wall. The mechanism of division remains unknown (31).

Morphogenetic Influence of Fibrillar Network

The surface tension forces should impose a spherical shape on protoplasts growing in liquid media. The presence of a firm fibrillar framework synthesized on the protoplast surface can change the isodiametric growth of yeast protoplasts. The increasing volume of the cytoplasm causes a break in the fibrillar network and a naked hernia is formed covered with plasma membrane only (34, 49, 71, 72). Under the light microscope these patterns look like budding of protoplasts. Time-lapse microcinematography has shown, however, that the herniae arise by sudden evagination, proving that the formation of a hernia is a physical process only. On the surface of the hernia new fibrils are synthesized. The same events can be repeated several times, resulting in long, periodically constricted formations which resemble pseudomycelial growth.

Thus, the presence of a fibrillar network determines the morphology of protoplast growth, but only as a physical factor. We can find an analogy to this process in nonliving systems, e.g., in the growth and morphology of Traube's vesicles. The fibrillar network in itself cannot replace the cell wall in its morphogenetic significance.

We must emphasize that the processes described above can simulate true budding or pseudomycelial growth when observed in the light microscope and might be considered as protoplast reversion. It is apparent that the reliable criteria of true reversion are the presence of a new cell wall, which can be proven only with the aid of electron microscopy and the isolation of revertants capable of further reproduction.

Again, a comparison with protoplasts of other plant cells shows that the incompletely built cell walls cannot take over the role of the normal cell wall in morphogenesis. The protoplasts of moulds (21), higher plant cells, and green algae (24) perish, although they have synthesized the fibrillar component of the cell wall. Only one exception is known (again, in bacteria), i.e., stable L-forms of the spheroplast type which can multiply despite having abberant cell walls on their surface.

Experimental Inhibition of Wall Synthesis

Cell wall formation on yeast protoplasts can be selectively and completely inhibited by adding snail enzymes to media suitable for wall regeneration. At the same time, the growth of protoplasts and nuclear division continue uninfluenced, and thus giant multinuclear forms arise (36, 104). If these growing protoplasts are transferred to enzyme-free media, structurally normal cell walls are formed within several hours. The length of the period during which cell wall regeneration is blocked by snail enzymes is not decisive. The blockade of cell wall regeneration is thus reversible at any time.

Different results are obtained when the reversibility of protoplast reversion is studied. If the protoplasts incubated in the presence of snail enzymes are transferred to the enzyme-free medium during the first 4 to 6 hr, the cell wall regenerates and the walled protoplasts revert then by budding to the original strain. If the blockade lasts longer than 6 hr, the protoplasts regenerate the cell wall after transfer to the enzyme-free medium, but no reversion of walled protoplasts takes place. Thus, it appears that in *S. cerevisiae* the inhibition of wall regeneration by snail enzymes is reversible at any time, instead of protoplast reversion only after a relatively short period of inhibition. This means that if synthesis of a normal wall is unduly delayed, morphogenesis is no longer feasible; i.e., budding does not occur and after several days the protoplasts die. There are no experimental data which might explain these findings. Evidently, some intracellular control processes responsible for the normal cell cycle and cytokinesis are irreversibly impaired if the synthetic processes making wall material are allowed to run to waste for a time without the feedback action of the wall as a morphological unit. In any case, this model system presents new possibilities for studies on the regulation of cell morphogenesis.

On the other hand, experimental inhibition of wall biosynthesis in protoplasts of *Schizosaccharomyces pombe* and *N. elongata* (36) has shown the reversibility both of wall regeneration and of reversion to be without relation to the period of inhibition. These protoplasts grow in the presence of snail enzymes to make giant multinucleate forms, and after transfer to enzyme-free media these are able to synthesize a new cell wall and they always revert. It is remarkable that all these protoplasts, unlike those of budding yeasts, can form new cell walls also in liquid media. This indicates that the different types of cell reproduction in yeasts is reflected not only in different control of the cell wall synthesis by protoplasts but also in different feedback mechanisms of the cell wall-cytoplasm relation during protoplast reversion.

The reversibility of the inhibition of cell wall formation has also been proved in protoplasts of *Rhizopus nigricans* (23), which normally regenerates the cell wall very fast in liquid media, and in protoplasts of *Fusarium culmorum* (25).

Cell Shape

When the yeast cell—and, by analogy, all other plant cells—is deprived of the cell wall, the protoplast assumes a spherical shape according to physical laws. This leads to the simple conclusion that the shape of a plant cell is determined principally by the three-dimensional architecture of the cell wall. Similarly, the morphology of the growth of naked protoplasts does not result from their intracellular structure but is influenced by physical factors only, such as environment or aberrant cell wall formation.

The shape of the new cell wall formed on the surface of spherical protoplasts corresponds always to the spherical form and not to the specific cell shape of the original strain. Yeast protoplasts, under the influence of external factors, may have a shape other than spherical; the morphology of the cell wall synthetized de novo follows the shape of the protoplast. Thus, if the protoplasts grow in a close packing in gelatin, their shape is deformed and the wall material is deposited in the fissures and free spaces left between the protoplasts, forming quite irregular envelopes. Often, only one wall layer is to be found between the two protoplasts in contact, forming a common wall for both (76, 78). It is apparent that in these cases the cell wall assumes the shape of the regenerating protoplasts.

When new cells originate from the walled protoplasts, by budding in budding yeasts or by forming a septum in fission yeasts, the cell wall is already the decisive factor determining the cell shape. It follows that the genetically controlled cell wall synthesis determines the cell shape only if the cytoplasm was not forced initially, by external factors, to assume another shape.

Cytokinesis

The nuclei continue to divide during the growth of *Saccharomyces cerevisiae* protoplasts in liquid and on the surface of solid nutrient media, or during the inhibition of cell wall synthesis, in all kinds of growing yeast protoplasts. This is not accompanied by the division of protoplasts. Evidently, the cause is the absence of the cell wall. From all observations, the multiplication of protoplasts without walls (61) and of protoplasts enveloped with a fibrillar network only cannot be

identified with cytokinesis. In such cases, the protoplasts are divided according to simple physical principles. Thus, for instance, if the length of a polar-growing protoplast exceeds 2 πr, its division into two sperical parts must occur according to the principle defined many years ago by Berthold (5). The subprotoplasts, originating from protoplasts coated with a fibrillar network through herniation, have already been described. The majority of protoplast parts (subprotoplasts) arising in this way are without a nucleus, which also proves that these processes cannot be regarded as a controlled cellular operation.

Immediately after the new cell wall is formed round the protoplasts, cytokinesis occurs of a type appropriate to the species. Budding in *S. cerevisiae* protoplasts is usually of a multipolar character (72). It occurs over a relatively wide base as compared with normal cells. The continuity of the mother wall is clearly broken and the cell wall of the bud is attached to the inner face of the protoplast wall over a wide area round the opening. The septum seems to be synthesized as an independent unit inserted into the base of the opening (78). It is not known whether the septum is formed as a single lamella, which later undergoes fission, or whether it is synthesized separately and simultaneously by the mother protoplast and the daughter cell. The structure of the resultant bud scars is essentially the same as in normal yeasts, but their diameter is larger (up to 4 μm). This observation on the budding of reverting protoplasts agrees with that reported by Nickerson (84) in normal budding cells, but does not support the view about the structural continuity of the walls of the mother and daughter cells (8, 60).

The renewal of normal cellular relations (i.e., the synchronization of karyokinesis with cytokinesis in walled protoplasts containing a larger number of nuclei) proceeds gradually in the next cell generation. The first generation of cells arising by budding of the newly walled *S. cerevisiae* protoplasts usually differs in shape from normal cells (73). The cells are larger, irregular, contain several nuclei, and often reveal multipolar budding (Fig. 9). The next generation of cells usually corresponds in size and shape to typical cells of the original strain. One can suggest that the reduction of the nuclear number per cell is due to the greater probability that one nucleus, rather than several, passed into the bud before it is

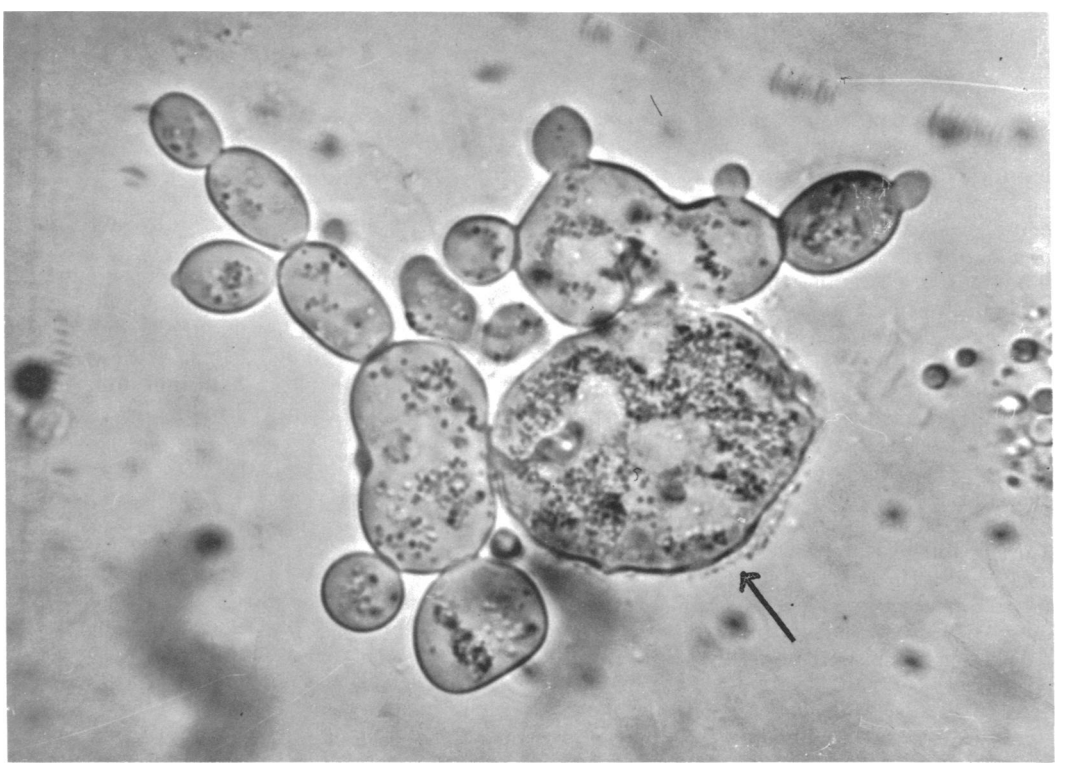

FIG. 9. *Reversion of walled Saccharomyces cerevisiae protoplast (arrow) by budding. Note the atypical shape and multipolar budding of the first cell generation.* × *400.*

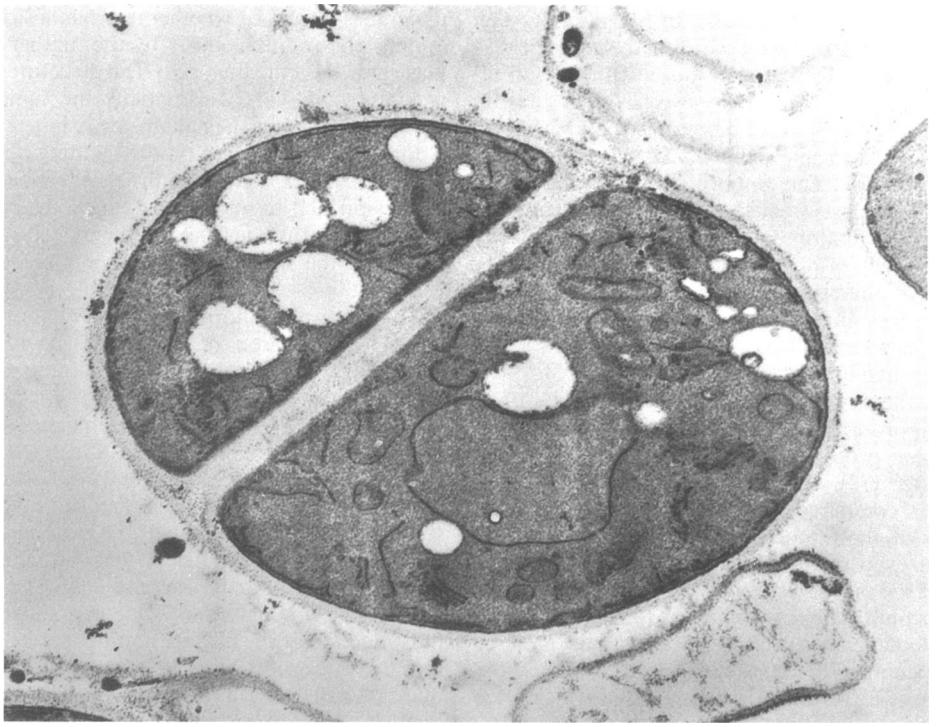

FIG. 10. *First step in the reversion of a protoplast of Schizosaccharomyces pombe. The protoplast enveloped by the regenerated cell wall divides into two cells by means of a transverse septum.* × *19,500. (Courtesy of Marie Havelková.)*

closed by the septum. This nuclear normalization is connected with a reduction of the cell size. Exceptions have been described in *S. cerevisiae*—i.e., revertants of normal size which contain several nuclei (65). It is interesting that these strains maintained these properties in the course of thousands of generations.

No nuclear division takes place in the course of cell wall regeneration in *Schizosaccharomyces pombe* protoplasts (33). The first nuclear division in the newly walled protoplast usually coincides with the formation of the transverse septum, which arises by centripetal growth. Thus, two mononuclear cells arise, their shape being completely atypical for *Schizosaccharomyces* (Fig. 10). Both cells form synchronously and through a process which is similar to budding—one mononuclear cell from each which corresponds in shape and size to typical *Schizosaccharomyces* cells. These cells divide further by normal fission.

The synchronization of nuclear division with protoplast reversion has been described in the apiculate yeast *N. elongata* (34). The morphology of reversion resembles budding taking place successively at opposite poles. The first generation of new cells is mononuclear. A detailed cytological analysis of this process has not been made; therefore, a comparison with the type of division in normal apiculate yeast cells as described by Streiblová (98) is impossible.

Comparative studies are needed to elucidate cytokinesis in reverting protoplasts of yeast species differing in the type of cell division. In spite of this, the above-mentioned examples show that the genetically determined type of cytokinesis is also reflected in the morphology of protoplast reversion. One can suggest that the studies of protoplast reversion may contribute to the knowledge of normal yeast cell reproduction and, particularly, to the role of the cell wall in the mechanism of cytokinesis.

CELL WALL, CELL INTEGRITY, AND REPRODUCTION

In conclusion, we can see that the experimentally prepared protoplasts of all lower plant cells, including probably the bacteria, are able to synthesize a new cell wall and revert to normal cells, cell wall regeneration and protoplast reversion being related to the continued existence of the cell. In budding yeasts, in some green algae (22), and in bacteria (53), wall formation must be facilitated by the creation of special environmental conditions. The morphology of reversion seems to depend on the proper balance between

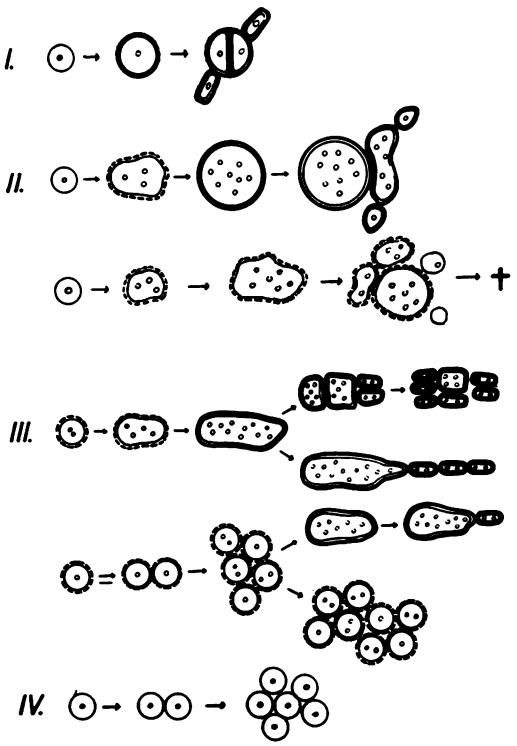

FIG. 11. *Fate of protoplasts and spheroplasts in different lower-plant cells. (I) Molds, some yeasts, bacteria (?); (II) budding yeasts; (III) spheroplasts of bacteria and L-forms of spheroplast type; (IV) stable L-forms of protoplast type. Key: plasma membrane, thin line; abberrant cell walls (fibrillar network), broken line; regenerated cell wall, thick line; nuclei small circles.*

the rate of wall regeneration and the rate of growth of the protoplast.

Four patterns of reversion are schematically summarized in Fig. 11. Type I is characterized by rapid wall regeneration and no karyokinesis during slow protoplast growth. The first nuclear division immediately precedes the reversion. This morphological type of reversion includes the protoplasts of moulds grown in liquid media [reviewed by Strunk (100)] and protoplasts of *Schizosaccharomyces*, *Nadsonia*, *Endomycopsis*, and probably *B. subtilis* grown in gelled media (53).

In type II, the protoplasts grow rapidly and the nuclei divide, but wall regeneration is relatively slow. This morphology is found in protoplasts of budding yeasts embedded in gelled media, in protoplasts of green algae after addition of pectins to the culture medium (22), and in all plant protoplasts in which the wall regeneration is, by experiment, reversibly inhibited. The protoplasts of budding yeast and green algae grown in liquid media synthesize only aberrant walls, and consequently never revert.

Type III is represented by bacterial spheroplasts and L-forms of the spheroplast type having remnants of the original cell wall. The spheroplasts easily recover the cell wall and revert; so do the unstable L-forms. The spheroplast type of stable L-forms multiply and do not revert, having only aberrant walls.

Stable L-forms of the protoplast type capable of reproduction without any cell wall structure represent the last group (type IV). Whether other plant cells deprived of their cell walls are, under special conditions of cultivation, capable of permanent reproduction in the protoplast state remains uncertain.

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