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RNA Synthesis and the Formation of the Cell Wall. Effect of Lomofungin on Regenerating Protoplasts of *Saccharomyces cerevisiae*

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While lomofungin at 20 μg ml^{-1} effectively halted the synthesis of ribonucleic acids in protoplasts of *Saccharomyces cerevisiae*, the onset of biogenesis of new wall on the protoplast surface was not impaired. The synthesis of mannan–protein wall matrix continued for about 30 min after addition of lomofungin while the formation of 1,3-β-glucan networks was not influenced even after prolonged incubation. These results suggest that the life-span of limiting mRNA molecules coding for the proteins of the wall matrix is about 30 min while the turnover of 1,3-β-glucan synthases is relatively low.

INTRODUCTION

When yeast protoplasts are cultivated in an osmotically stabilized, liquid nutrient medium, they synthesize on their surface a dense fibrillar network of 1,3-β-glucan (Kreger & Kopecká, 1973, 1976*a*, *b*; Nečas, 1965; Kopecká *et al.*, 1967; Eddy & Williamson, 1959), while the mannan–protein wall constituents, including some extracellular enzymes, diffuse into the surrounding medium (Nečas & Svoboda, 1967; Lampen, 1968; Svoboda & Nečas, 1970; Farkaš *et al.*, 1973; Kreger & Kopecká, 1973, 1976*a*, *b*). With *Saccharomyces cerevisiae* protoplasts, complete regeneration of the wall occurs only in a medium of high viscosity, such as 30% gelatin (Nečas, 1961) or 2% agar (Svoboda, 1966). Gels probably facilitate the completion of the newly formed wall by preventing the diffusion of the precursors of the wall matrix material into the surrounding medium (Nečas & Svoboda, 1967) and thus promoting cross-linking of individual mannan–protein molecules at the protoplast surface (Nečas, 1971).

The use of various inhibitors can contribute to a better understanding of how the formation of the individual wall components is interrelated and regulated. The use of inhibitors of protein synthesis, such as cycloheximide (Nečas *et al.*, 1968), and of mannan formation, such as 2-deoxyglucose or tunicamycin (Farkaš *et al.*, 1969, 1970; Kuo & Lampen, 1974), has shown that the formation of the mannan–protein wall component in yeast protoplasts is dependent on undisturbed protein synthesis while the formation of wall glucan is largely independent of protein synthesis in yeast protoplasts.

The inability of anucleate protoplasts to regenerate a new wall (Kopecká *et al.*, 1974) has directed our attention towards the role of RNA synthesis in the biogenesis of the wall, raising the question of whether the initiation of wall regeneration is dependent on the

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synthesis of new molecules of RNA directing the synthesis of enzymes and proteins necessary for the initiation of wall formation. 5-Fluorouracil, widely used as an inhibitor of RNA synthesis, is ineffective in yeast protoplasts, and has no effect on the regeneration of the wall (Sošková *et al.*, 1970).

In the present study with yeast protoplasts we used lomofungin, a potent inhibitor of the synthesis of high molecular weight RNA (messenger RNA and ribosomal RNA) in yeast (Kuo *et al.*, 1973; Cannon *et al.*, 1973; Fraser *et al.*, 1973). Lomofungin interacts as a complex-forming agent with RNA polymerases (Cano *et al.*, 1973; Ruet *et al.*, 1975). By means of studies of radioactive precursor incorporation and light and electron microscopy, we have investigated the effect of blocking RNA synthesis on the regeneration of the wall in protoplasts of *S. cerevisiae*.

METHODS

Yeast. Saccharomyces cerevisiae strain CCY 21-4-59 (Czechoslovak Collection of Yeast and Yeast-like Microorganisms, Institute of Chemistry, Slovak Academy of Sciences, Bratislava) was used throughout. The yeast was cultivated in liquid YNBG medium pH 5·5 (Johnson, 1967).

Preparation of protoplasts. Protoplasts were prepared from exponentially growing yeast by the method of Eddy & Williamson (1959). Following release, the protoplasts were washed free from snail enzymes with 0·8 M-mannitol in 0·05 M phosphate/citrate buffer pH 5·5 and immediately used in the experiments.

Lomofungin (kindly provided by Dr G. B. Whitfield, Upjohn Co., U.S.A.) was dissolved either in dimethyl sulphoxide (DMSO) or in water adjusted to pH 8 with KOH. The stock solution (2 mg ml^{-1}) was stored at 0 °C.

Determination of sensitivity of cells and protoplasts to lomofungin. Cells growing exponentially in YNBG medium were distributed into flasks containing various amounts of lomofungin and growth was followed either by measuring the absorbance at 650 nm or by counting the cells in a Bürker chamber. Colony-forming units were determined by cultivating cells on agar plates. Lomofungin at 20 μg ml^{-1} was found to be sufficient for complete inhibition of growth.

Effect of lomofungin on protein and RNA synthesis in protoplasts. Protoplasts were suspended in two 12 ml portions of osmotically stabilized YNBG medium at a concentration of 1×10^7 ml^{-1}. After 15 min incubation at 30 °C radioactive markers, [U-^{14}C]uracil (5 mg, 10 μCi) and/or L-[U-^{14}C]valine (5 mg, 10 μCi), were added. Immediately after addition of the markers and at appropriate intervals thereafter, 0·5 ml samples were withdrawn from the protoplast suspension pipetted into test tubes containing 0·5 ml of cold 10 % (w/v) trichloroacetic acid (TCA) and stored at 0 °C. After 1 h incubation, each protoplast suspension was divided into two parts: lomofungin was added to one flask at a final concentration of 20 μg ml^{-1}, and a corresponding amount of DMSO was added to the other (control). Incubation was then continued and samples were withdrawn from these suspensions. After incubation was completed, the samples were centrifuged, washed three times with cold 5 % (w/v) TCA and finally with cold absolute ethanol. The pellets were dissolved in 0·2 ml tissue solubilizer (NCS, Nuclear Chicago) and counted with 5 ml toluene scintillation liquid in a scintillation spectrometer (Packard Tri-Carb, model 3330).

Effect of lomofungin on regeneration of the wall in protoplasts. The formation of the fibrillar wall component was followed in osmotically stabilized, liquid YNBG medium containing lomofungin (20 μg ml^{-1}). In some experiments the protoplasts were treated for 60 min with lomofungin in 0·8 M-mannitol before transfer to the nutrient medium.

The regeneration of the complete wall was followed in 30 % (w/v) gelatin (Nečas, 1961) dissolved in N-1 nutrient medium (Kelleti *et al.*, 1954). The protoplasts were embedded in warm (36 °C) liquefied gelatin either directly or after incubation with lomofungin in 0·8 M-mannitol or in YNBG medium with mannitol.

Pretreatment of cells with lomofungin. In some experiments the cells were preincubated with lomofungin (20 μg ml^{-1}) in YNBG medium for 30, 60 or 90 min before conversion to protoplasts, or for 30, 60 or 90 min during their conversion to protoplasts.

Electron microscopy. Suspensions of protoplasts were transferred to Formvar-coated grids and washed by the water droplet method. The preparations were shadowed with platinum and carbon and examined in an electron microscope (Tesla, type BS 500).

The protoplasts embedded in gelatin were liberated by heating the medium to 37 °C, diluted with water and washed. The cytoplasm was removed either by washing the protoplast pellet with 0·5 % (w/v) lauryl sulphate at 50 °C (Kopecká, 1976*a*) or by incubation with trypsin (Svoboda & Nečas, 1970).

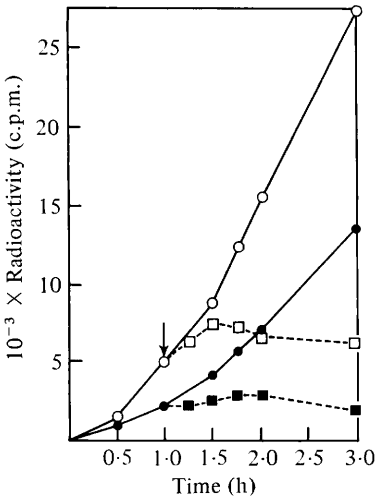


Fig. 1. The effect of lomofungin on protein and RNA synthesis in protoplasts of *S. cerevisiae*. Protein synthesis in control protoplasts (○) and in lomofungin-treated protoplasts (□) was indicated by L-[^{14}C]valine incorporation. RNA synthesis in control protoplasts (●) and in lomofungin-treated protoplasts (■) was indicated by [^{14}C]uracil incorporation. The arrow indicates the time at which lomofungin was added.

RESULTS

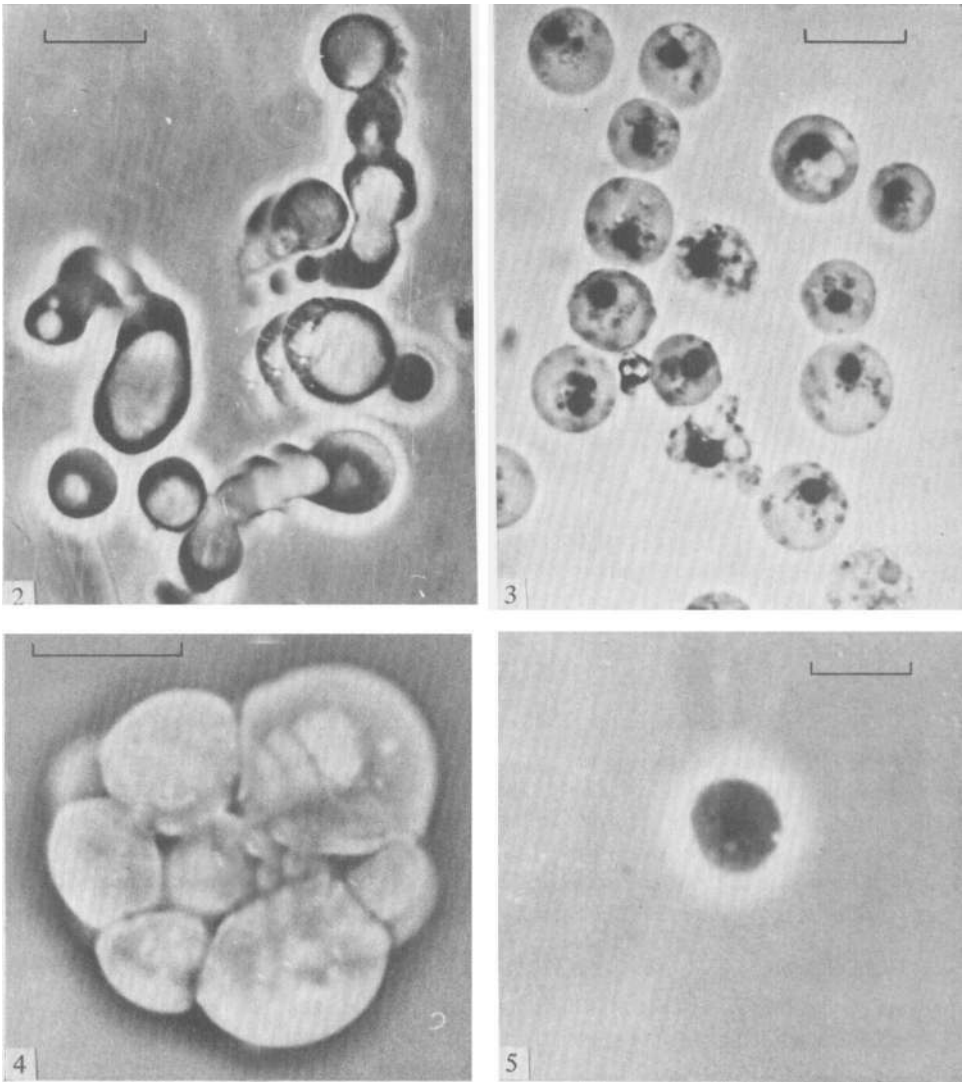
Effect of lomofungin on RNA and protein synthesis in protoplasts

The kinetics of incorporation of the radioactive precursors uracil and L-valine into the RNA and protein fractions, respectively, are shown in Fig. 1. The addition of lomofungin at a concentration of 20 μg ml^{-1} to protoplasts cultures caused almost immediate inhibition of RNA synthesis, while the formation of protein was scarcely affected for at least 30 min. The slow decrease in the radioactivity of RNA and protein fractions observed in the experiment could be explained by their decay due to turnover.

Viability and fate of protoplasts incubated with lomofungin in liquid medium

The aim of these experiments was to determine whether the protoplasts maintain their viability in the period before the onset of biogenesis of the wall in the presence of lomofungin. The protoplasts grown in the presence of lomofungin were mostly spherical or ovoid (Fig. 3); lomofungin obviously inhibited the polarized growth of protoplasts. During cultivation of protoplasts in the presence of lomofungin the nuclei became coloured due to selective binding of the drug (Fig. 3), as also occurs with intact cells (Kopecká, 1976*b*) and with other organisms (Kopecká & Gabriel, 1978). The protoplasts cultivated in the presence of lomofungin usually contained only one nucleus, rarely two. This observation indicates that nuclear division does not occur in protoplasts inhibited by lomofungin. During incubation in liquid medium with lomofungin, approximately 10 to 20% of protoplasts lysed during the first 60 min. After 4 h cultivation the fraction of lysed protoplasts reached 30%, and after 10 h all the protoplasts had lysed.

In contrast, lysis in the controls lacking lomofungin was not usually observed until after 4 h. The protoplasts incubated in liquid medium without lomofungin exhibited polarized, ovoid or spherical growth (Fig. 2). In these protoplasts usually two or three nuclei were detected after 105 min cultivation (as indicated by staining the protoplasts with lomofungin).



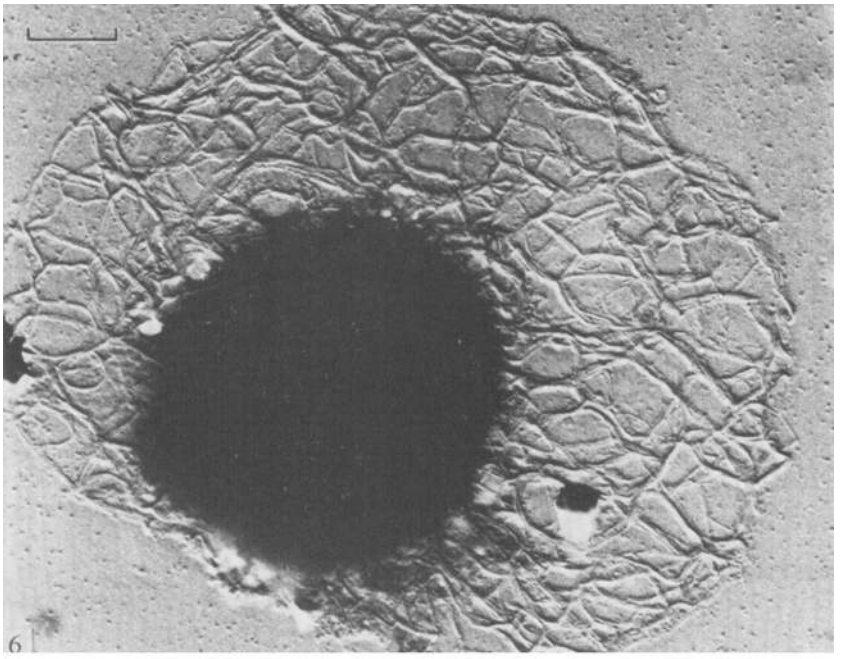
Bar markers represent 10 μm.

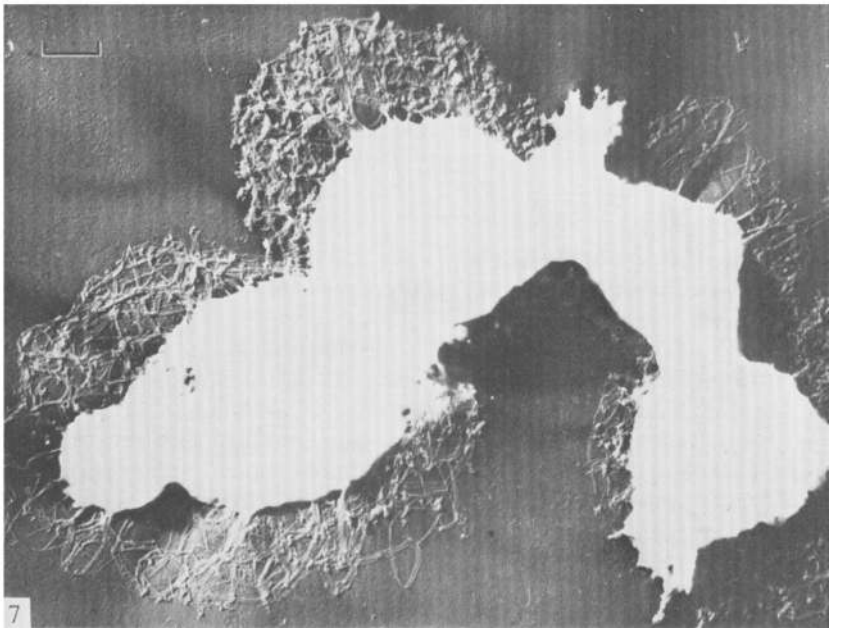
Fig. 2. Control protoplasts of *S. cerevisiae* cultivated for 4 h in YNBG medium with mannitol and DMSO (10 μl ml^{-1}), but without lomofungin. Protoplasts grew apically; some of them are spherical or ovoid.

Fig. 3. Protoplasts of *S. cerevisiae* incubated for 60 min in YNBG medium with mannitol and DMSO (10 μl ml^{-1}) containing lomofungin (final concn 20 μg ml^{-1}). Protoplasts are intact and spherical, and their nuclei are stained red by lomofungin. In some protoplasts vacuoles are visible.

Fig. 4. Complete wall regeneration in control protoplasts of *S. cerevisiae*. Protoplasts were washed free of snail enzymes using 0·8 M-mannitol, inoculated in YNBG medium with mannitol and DMSO (10 μl ml^{-1}) and immediately inoculated into 30 % (w/v) gelatin/N-1 medium with DMSO at 36 °C and cultivated for 20 h at room temperature. Wall regeneration in the protoplasts was followed by reversion to normal cells. A microcolony of a reverting protoplast is visible inside the gelatin medium.

Fig. 5. Inhibition of wall regeneration in protoplasts of *S. cerevisiae*. Protoplasts were suspended in YNBG medium with mannitol and DMSO (10 μl ml^{-1}) containing lomofungin (final concn 20 μg ml^{-1}). At time zero, they were inoculated into 30 % gelatin/N-1 medium containing lomofungin. After 20 h cultivation, the protoplasts were still alive and spherical, but reversion to normal cells did not occur.





Bar markers represent 1 μm. Metal shadowed preparations.

Fig. 6. The fibrillar network of a protoplast cultivated for 20 h in YNBG medium with mannitol and DMSO (10 μl ml^{-1}) containing lomofungin (final concn 20 μg ml^{-1}).

Fig. 7. The fibrillar network of a control protoplast cultivated for 20 h in YNBG medium with mannitol and DMSO (10 μl ml^{-1}), without lomofungin. Fibrillar nets are not spherical, as in Fig. 6, but tubular.

Regeneration of protoplasts in the presence of lomofungin in 30% *gelatin*

Light microscopy of protoplasts cultivated in 30% gelatin revealed that in the absence of lomofungin the regeneration of walls was complete after 10 h at room temperature (Nečas, 1965). The protoplasts then reverted into cells which could be detected as microcolonies around the regenerated protoplast (Fig. 4). However, the protoplasts cultivated in the presence of lomofungin did not revert (Fig. 5) and remained spherical even after 20 h cultivation in 30% gelatin. Nevertheless, unlike the protoplasts cultivated with lomofungin in liquid medium, they did not lyse.

Effect of lomofungin on the formation of 1,3-*β-glucan microfibrillar wall component in liquid medium*

Protoplasts of *S. cerevisiae* cultivated in osmotically stabilized, liquid YNBG medium containing lomofungin (20 μg ml^{-1}) formed isodiametric or ovoid microfibrillar nets on their surfaces (Fig. 6), but control protoplasts produced both spherical and tubular forms of microfibrillar networks (Fig. 7). The formation of elongated forms of networks is probably due to the continued growth of the protoplasts, a process which is inhibited in the presence of lomofungin (Fig. 3). However, the ultrastructure of microfibrils and the texture of whole networks was the same in both the controls and the protoplasts treated with lomofungin.

In further experiments we tried to block RNA synthesis with lomofungin before onset of the synthesis of 1,3-β-glucan microfibrils. The washed protoplasts were preincubated for 60 min in 0·8 M-mannitol with lomofungin and then transferred to nutrient medium containing lomofungin. [It should be noted that protoplasts do not form fibrils on their surface in the absence of a metabolizable carbon source (Kopecká *et al.*, 1968, 1970).] We found that, even after pretreatment with lomofungin, protoplasts could still synthesize fibrillar glucan on their surface.

Attempts to prepare protoplasts from cells pretreated with lomofungin failed. The majority of the cells lysed during their conversion to protoplasts with snail enzymes.

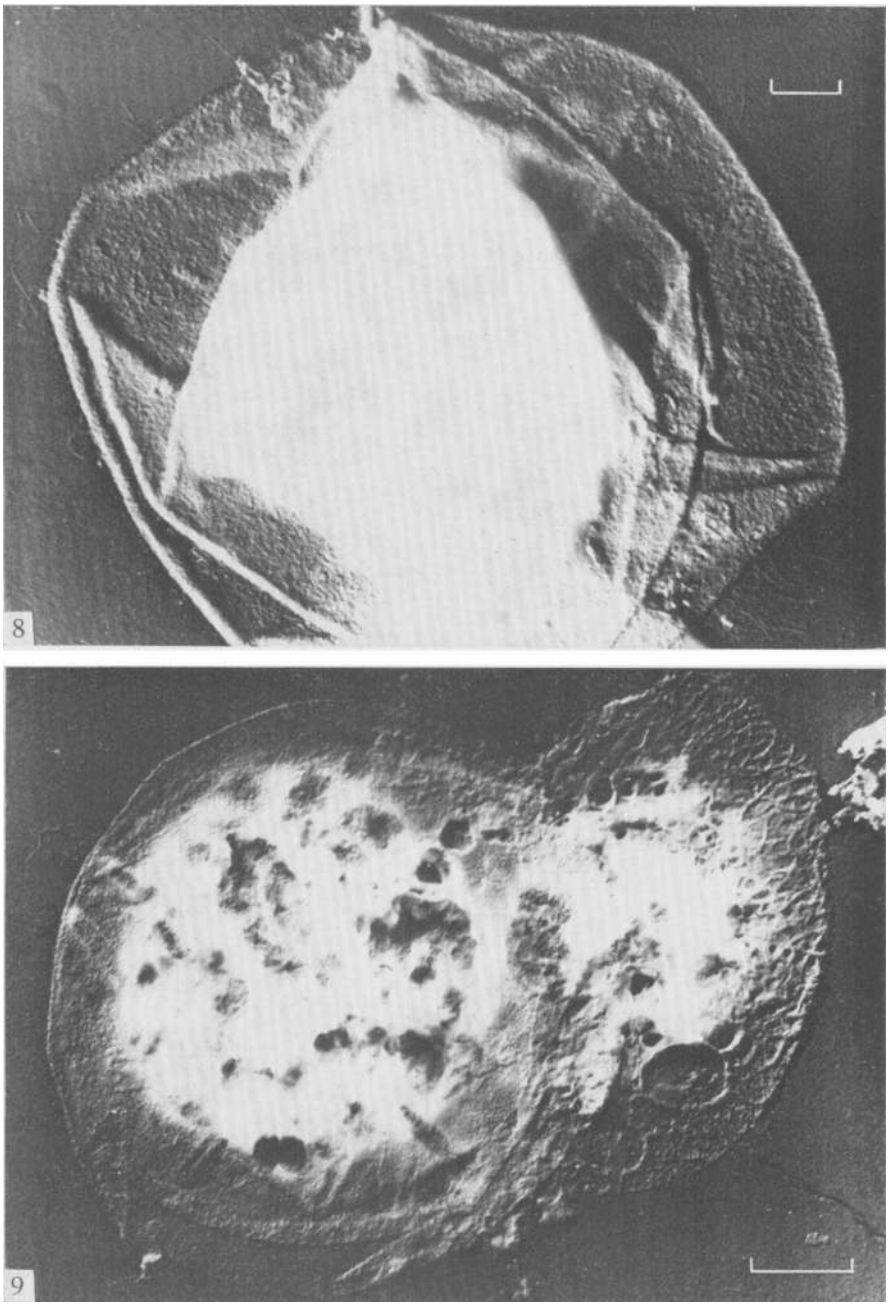
Effect of lomofungin on regeneration of the wall

The observation that lomofungin inhibits the reversion of protoplasts to normal cells raised the question of whether lomofungin interferes with the formation of some special wall component.

Washed protoplasts were suspended at a concentration of 1×10^7 ml^{-1} in osmotically stabilized YNBG medium containing lomofungin (20 μg ml^{-1}) and quickly transferred to liquefied 30%(w/v) gelatin containing N-1 medium and lomofungin (20 μg ml^{-1}). Electron microscopy of protoplasts cultivated in such medium revealed that thin walls composed of both microfibrils and amorphous components were formed on their surface (Fig. 8).

When protoplasts suspended in YNBG medium were treated with lomofungin 30 min before embedding in gelatin, only very thin walls were formed on their surface (Fig. 9). These walls again contained both amorphous and fibrillar components, but the amount of the former was less than in walls of protoplasts that had not been exposed to lomofungin before embedding in gelatin (Fig. 8). When protoplasts suspended in YNBG medium were treated for 60 min before embedding in gelatin, only the fibrillar component was formed on their surface (Fig. 10). The same result was obtained with protoplasts preincubated for 120 min with lomofungin.

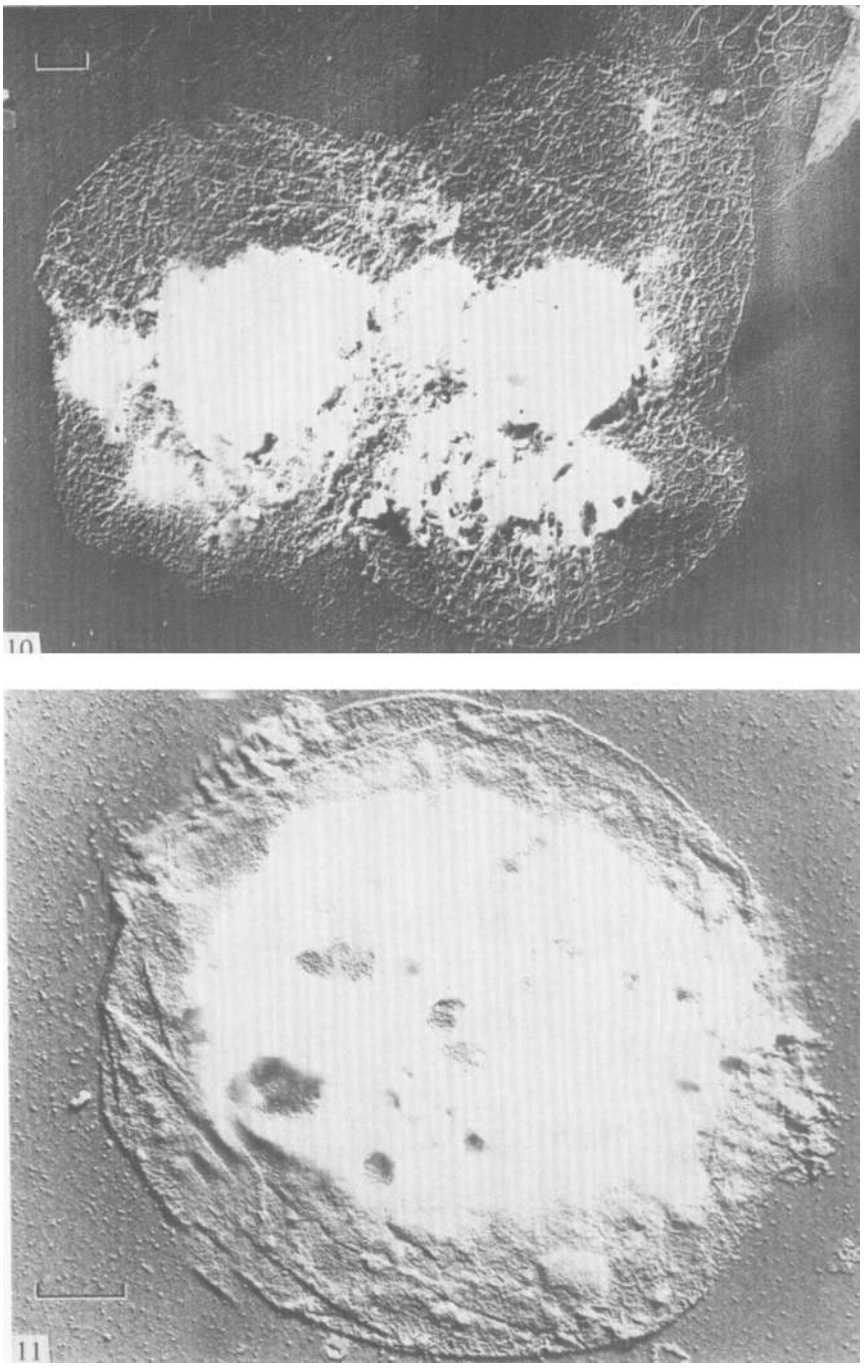
Similar results were also obtained with protoplasts preincubated with lomofungin in 0·8 M-mannitol instead of in the complete nutrient medium. As already mentioned, protoplasts cannot initiate wall synthesis in solutions lacking nutrients (see, for example, Nečas, 1971). During 30 min preincubation, lomofungin completely penetrated the protoplasts and coloured their nuclei. When these protoplasts were embedded in gelatin medium



Bar markers represent 1 μm. Metal shadowed preparations.

Fig. 8. The wall of a protoplast of *S. cerevisiae* suspended in YNBG medium with mannitol and DMSO (10 μl ml^{-1}) containing lomofungin (20 μg ml^{-1}), and immediately inoculated into 30 % gelatin/N-1 medium containing lomofungin (20 μg ml^{-1}) and incubated for 20 h. A thin wall containing an amorphous matrix is visible; fibrillar texture is also detectable.

Fig. 9. The wall of a protoplast of *S. cerevisiae* cultivated in YNBG medium with mannitol and lomofungin (20 μg ml^{-1}) for 30 min, then inoculated into 30 % gelatin/N-1 medium containing lomofungin (20 μg ml^{-1}) and incubated for 20 h. Very thin membranes containing both fibrils and amorphous material are visible.



Bar markers represent 1 μm. Metal shadowed preparations.

Fig. 10. The fibrillar network of a protoplast of *S. cerevisiae* cultivated in YNBG medium with mannitol and lomofungin (20 μg ml^{-1}) for 60 min, then inoculated into 30 % gelatin/N-1 medium containing lomofungin (20 μg ml^{-1}) and incubated for 20 h. Fibrils but no amorphous material are visible.

Fig. 11. The wall of a protoplast of *S. cerevisiae*, which had been washed free of snail enzymes in 0·8 M-mannitol, incubated in 0·8 M-mannitol containing lomofungin (20 μg ml^{-1}) for 30 min at 28 °C (lomofungin was visible in the nuclei at this stage), then inoculated into 30 % gelatin/N-1 medium containing lomofungin and incubated for 20 h. Thin walls composed of microfibrils and amorphous material are visible.

containing lomofungin, they formed surface walls containing not only fibrils but also amorphous material (Fig. 11).

DISCUSSION

The essential role of the nucleus in the regeneration of the wall was established by Sachs (1882), who studied the regeneration of plasmatic fragments of *Vaucheria*, and was later confirmed in a number of studies (e.g., Klebs, 1887, 1888; Nečas, 1956). Electron microscopic studies with anucleate yeast protoplasts (Kopecká *et al.*, 1974) have shown that these are unable to synthesize either the fibrillar or the amorphous wall components on their surface. It might be assumed that the role of the nucleus in wall regeneration is to form RNA coding for the enzymes and proteins involved in the formation of the wall. If this assumption were correct, the blocking of RNA synthesis should eventually inhibit wall formation.

In the present study we found that although lomofungin instantly and efficiently blocks RNA synthesis, it does not inhibit the initiation of wall regeneration in yeast protoplasts. In contrast to the effect of cycloheximide (Nečas *et al.*, 1968), the inhibitory effect of lomofungin on the synthesis of wall mannan–protein is delayed by about 30 min.

These findings indicate that the messenger RNA molecules necessary for the initiation of wall regeneration and the synthesis of wall matrix are already present in the fresh protoplasts. It is highly probable that these molecules of mRNA were synthesized as the normal products of the cell cycle while the yeast cells used for the preparation of protoplasts were still intact. This implies that synthesis of both the fibrillar and the amorphous wall components is of a constitutive rather than inductive nature.

The fact that the synthesis of the amorphous mannan–protein wall component is inhibited only after a delay may mean that mRNA molecules responsible for the formation of external glycoproteins break down rather slowly. Our observation that lomofungin, after 30 min, totally inhibits the formation of mannan–protein wall material is consistent with the finding of Kuo *et al.* (1973) who observed that the formation of yeast invertase, a mannan–protein, was inhibited 30 min after the addition of lomofungin. The blocking of α-glucosidase and acid phosphatase formation occurred about 20 to 30 min after the inhibition of RNA synthesis by lomofungin. Surdin-Kerjan & Robichon-Szulmajster (1975) determined the half-life of mRNA specific for homocysteine reductase to be 15 ± 1 min. The average half-life of the synthesis of protein in yeast in the presence of lomofungin was determined by Cannon *et al.* (1973) to be 20 to 25 min; this value is in agreement with the findings of other workers using different methods (Hutchinson *et al.*, 1969; Tønnesen & Friesen, 1973). Elorza *et al.* (1976) found that a temperature-sensitive mutant of *S. cerevisiae* did not synthesize RNA at the non-permissive temperature, although it continued to synthesize wall glucan and mannan. They explained their findings by the presence of mRNAs coding for the synthesis of peptide precursors of wall mannoproteins in the restricted yeast, the assumption being made that the turnover of the corresponding polysaccharide synthases is low.

The low turnover of polysaccharide synthases would allow the continuous synthesis of the wall polysaccharides, glucan and mannan, throughout the cell cycle in *S. cerevisiae* (Sierra *et al.*, 1973). It cannot be excluded, however, that the eventual decrease in protein synthesis caused by lomofungin is due to factors other than inhibition of RNA synthesis (Kuo *et al.*, 1973; Cannon *et al.*, 1973).

In the present work we have found that lomofungin, like cycloheximide (Nečas *et al.*, 1968), is ineffective in preventing the formation of glucan microfibrils in yeast protoplasts. The glucan microfibrils are formed independently of the synthesis of RNA and proteins. A likely explanation for this is that glucan synthases have a relatively low turnover and remain functional when their synthesis is prevented.

That the inability of anucleate protoplasts to synthesize wall mannan–proteins may be

due to the failure of protein and RNA synthesis is indicated by our studies of the incorporation of [^{14}C]valine and [^{14}C]uracil (unpublished results). These latter findings, however, do not explain the inability of anucleate protoplasts to synthesize fibrillar wall glucan (Kopecká *et al.*, 1974).

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