ELECTRON MICROSCOPY OF ULTRATHIN SECTIONS OF *SCHIZOSACCHAROMYCES OCTOSPORUS*

III. Ascosporogenesis, Ascospore Structure, and Germination

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Investigations of the yeast cell by means of ultrathin sectioning and electron microscopy, especially when correlated with other studies by light microscopy, have given the field of yeast cytology added scope and dimension. The majority of these studies have, however, been carried out with *Saccharomyces cerevisiae* (Agar and Douglas, 1957; Hashimoto, *et al.*, 1958*a*, *b*, 1959). More recent studies in this laboratory have been concerned primarily with *Schizosaccharomyces octosporus*. Light and electron microscopic observations of the cytological changes occurring during cell division and conjugation (Conti and Naylor, 1959, 1960) have already been described. The present investigation of ascosporogenesis and germination completes the preliminary study of the life cycle of *S. octosporus* by means of electron microscopy.

The process of ascospore formation ("free cell formation") has been described by Guilliermond (1901, 1920) and Widra and DeLamater (1955) as occurring by the condensation of protoplasm around each of the nuclei, with subsequent formation of the spore wall. The process of germination was observed by these authors as being initiated by swelling of the ascospores. Guilliermond (1920) also reported that in most cases the spores elongate during germination and directly give rise to vegetative cells. These processes were followed by means of electron microscopy to observe cytological changes beyond the resolution limit of the light microscope. The fate of the vacuole, and behavior of the nucleus and other cellular components, was also followed.

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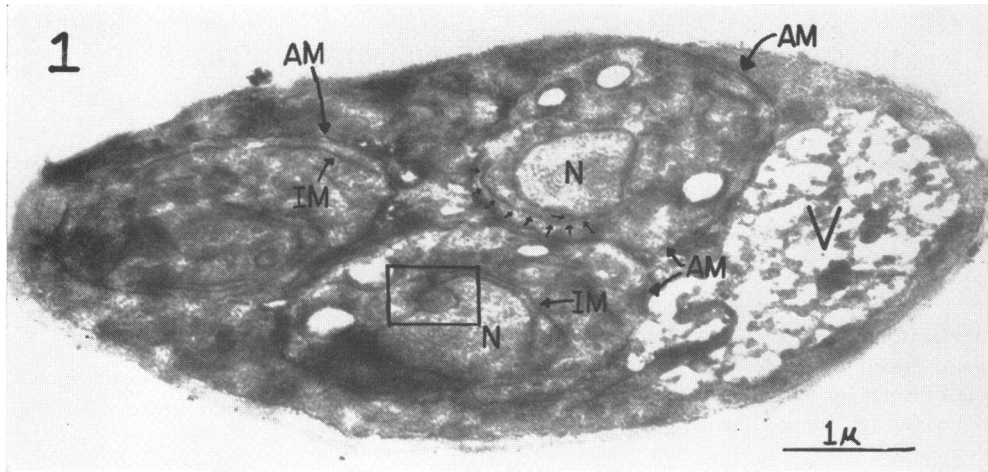
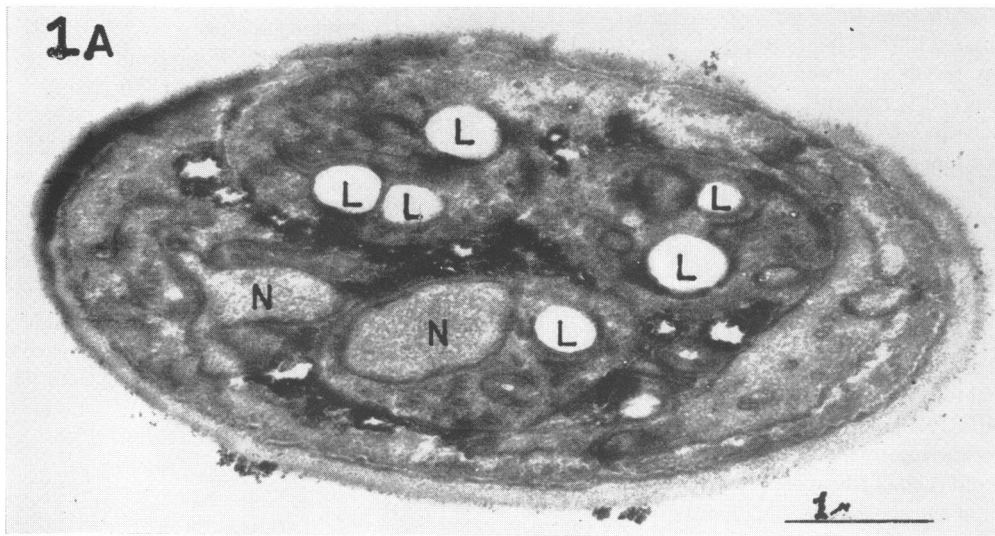
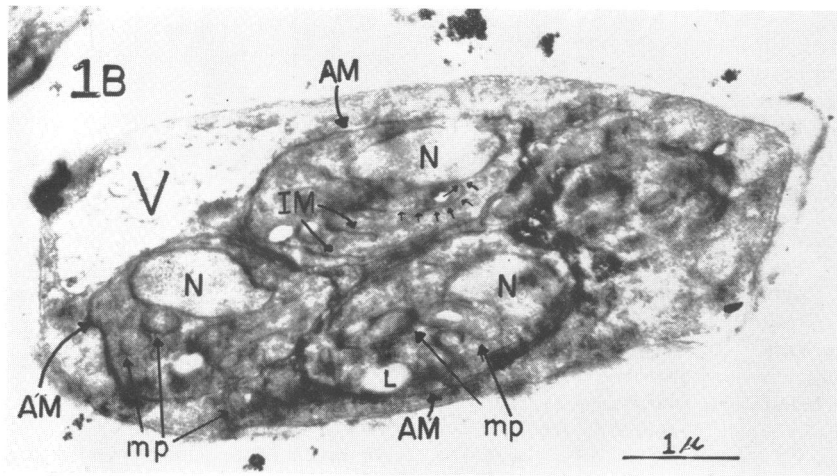
MATERIALS AND METHODS

Culture techniques. S. octosporus strain NRRL Y-854, also employed in our previous studies, was obtained from Dr. L. J. Wickerham. Cells were routinely grown in a glucose-yeast extract medium (glucose, 1.0 per cent; yeast extract, 2.0 per cent; peptone, 0.5 per cent; KH_2PO_4, 0.1 per cent and $MgSO_4$, 0.05 per cent). The pH of the medium was adjusted to 7.0 before autoclaving. Cells in intermediate stages of sporulation were obtained by inoculating 24-hr-old cells on to YM agar (glucose 1.0 per cent; malt-extract, 0.3 per cent; yeast extract, 0.3 per cent; agar, 2.0 per cent, pH unadjusted), and incubating at 28 to 30 C. Sporulation was essentially complete within 48 hr. Cells in various stages of ascospore formation were obtained by collecting cells between 15 and 32 hr after inoculation. Ascospores were obtained in greater than 95 per cent yield within 3 to 5 days. Ascospores were germinated by transfer to the glucose-yeast extract medium and incubation at 30 C with vigorous aeration. The degree of synchrony of sporulation and germination was satisfactory for electron microscopic investigation.

Specimen preparation. The techniques used for specimen preparation were the same as those previously described (Conti and Naylor, 1959). Cells were fixed in a 1.5 per cent aqueous solution of $KMnO_4$, dehydrated by passage through a graded alcohol series, and embedded in butyl methacrylate. Partially polymerized methacrylate, and a polymerization temperature of 56 to 60 C were employed. All steps, except for the polymerization, were carried out at 4 C. Ultrathin sections (less than 0.1 μ) were obtained by means of a Porter-Blum microtome equipped with a glass knife, and the technique of Satir and Peachey (1958) employed to decrease compression artifacts. Specimens were examined in an







FIGS. 1, 1A, and 1B

RCA-EMU 2B electron microscope equipped with a 50 or 100 μ objective aperture.

Cytochemical techniques and light microscopy. The processes of sporulation and germination were also followed by means of phase microscopy. Staining with dilute Lugol's solution, and Sudan black B were employed, respectively, for the demonstration of vacuoles and lipoidal inclusions.

RESULTS

The observations by light microscopy were in general agreement with the previous observations by Guilliermond (1901, 1920) and Widra and DeLamater (1955). It was also noted that ascus vacuoles, when present, were not enclosed within the developing or mature ascospore. Similarly, vacuoles were neither observed in germinating ascospores nor in fully developed vegetative cells. This latter observation is in agreement with the previous observations of Ganesan and Swaminathan (1958) and Conti and Naylor (1959). Lipoidal inclusions were observed within mature ascospores; however, their number appeared to decrease considerably during germination.

Electron microscopy. Figures 1 to 6 are electron micrographs of *S. octosporus* in various stages of ascospore formation, germination, and division. The criteria for identification of various structures from electron micrographs have been discussed in earlier reports (Hashimoto *et al.*, 1958*a*, *b*, 1959; Conti and Naylor, 1959, 1960).

Ascospore formation. Previous studies (Conti and Naylor 1960) indicated that nuclear division, subsequent to fusion nucleus formation, results in the formation of 8 nuclei, each surrounded by a double limiting membrane. The first indication of ascospore formation is the appearance of a double membrane (*AM*,[3] figures 1 and 1*B*) around each nucleus. Structures with circular or elliptical profiles, surrounded by a double membrane, were also encased within this membrane. Lipoidal inclusions, and an internal membrane system are also apparent within the developing ascospores. The origin of the ascospore membrane could not be established. Although this structure may be synthesized *de novo*, the possibility exists that it may have been derived from the preexisting internal membrane system (endoplasmic reticulum). Indeed, the electron micrographs indicate that the ascospore membranes around each nucleus initially are connected. There are also indications that the outer edge of the nuclear membrane is at least temporarily connected to a portion of the internal membrane system. The nuclei appear to be much less electron dense than the spore plasma, and are surrounded by a double membrane. It is of particular significance that the vacuoles appear to be excluded from the developing ascospore.

The next stage in ascospore development appears to be the formation of the ascospore wall. This structure initially appears as a narrow, nonelectron-dense area surrounding the ascospore membrane. This spore wall gradually increases in thickness thereby giving the mature ascospores a characteristic structure and appearance (figure 2). The cytoplasm not encased within the developing ascospores then appears to disintegrate. Overexposure of some of the micrographs indicate that the ascospore wall is comprised of at least two layers.

Electron micrographs (figure 3) also indicate that some spherical inclusions surrounded by a

[3] *AG* = ascus granules; *AM* = ascospore membrane; *AW* = ascus wall; *CM* = cytoplasmic membrane; *CW* = cell wall; *I* = invagination of the cell wall; *IM* = internal membranes; *L* = lipoidal inclusions; *mp* = mitochondrialike bodies; *N* = nucleus; *np* = nuclear "pores"; *SW* = ascospore wall; SW_1 = outer, electron dense ascospore wall; SW_2 = inner, nonelectron dense ascospore wall; *V* = vacuole.

Figures 1, 1A, and 1B. Immature ascospores prior to formation of the spore wall. Almost all of the nuclei are already surrounded by a double ascospore membrane. One ascospore (left, figure 1*A*) appears to be in the process of ascospore membrane formation. The nuclei are surrounded by limiting membranes, and appear to be less electron-dense than the cytoplasm. Structures with circular or elliptical profiles and surrounded by double membranes can be observed within the ascospores and ascus cytoplasm. Internal membranes can also be observed (figures 1 and 1*A*). Note particularly that the vacuole is excluded from the developing ascospore.

The apparent extension of the nuclear membrane from or to the ascospore cytoplasm (arrows, figure 1 and 1*B*) can also be observed. Figure 1 illustrates the appearance of a structure (in center of outlined area) which appears to be connected with the nuclear membrane. Identification of this structure is discussed in the text.

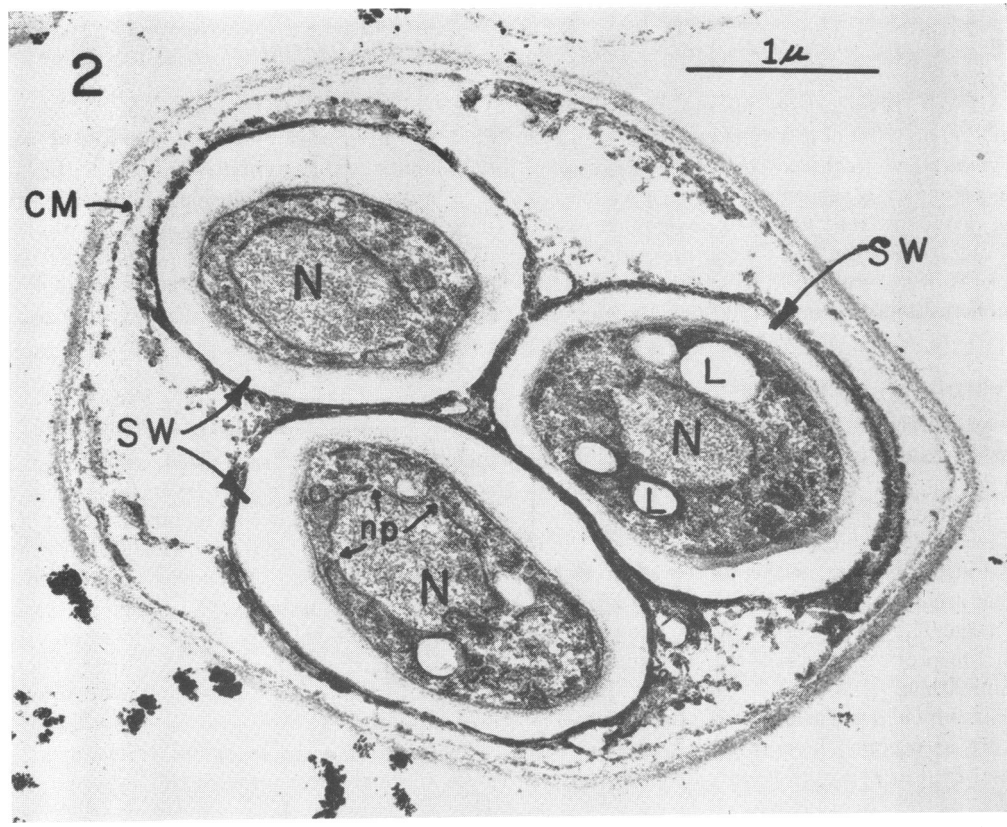


Figure 2. A cross section of a portion of a mature ascus. The ascospore walls are fully formed, and the ascus cytoplasm has almost completely disappeared. The ascus cytoplasmic membrane appears to be in the process of disintegration.

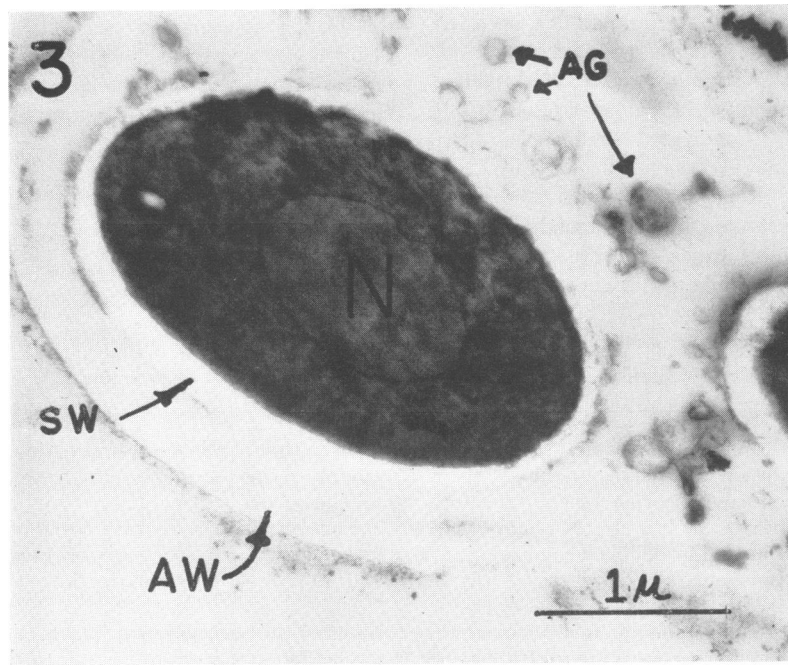


Figure 3. An overexposed micrograph of a section of a mature ascus illustrating the appearance of the ascus granules. Note again the low electron density of the ascus wall and the ascospore wall.

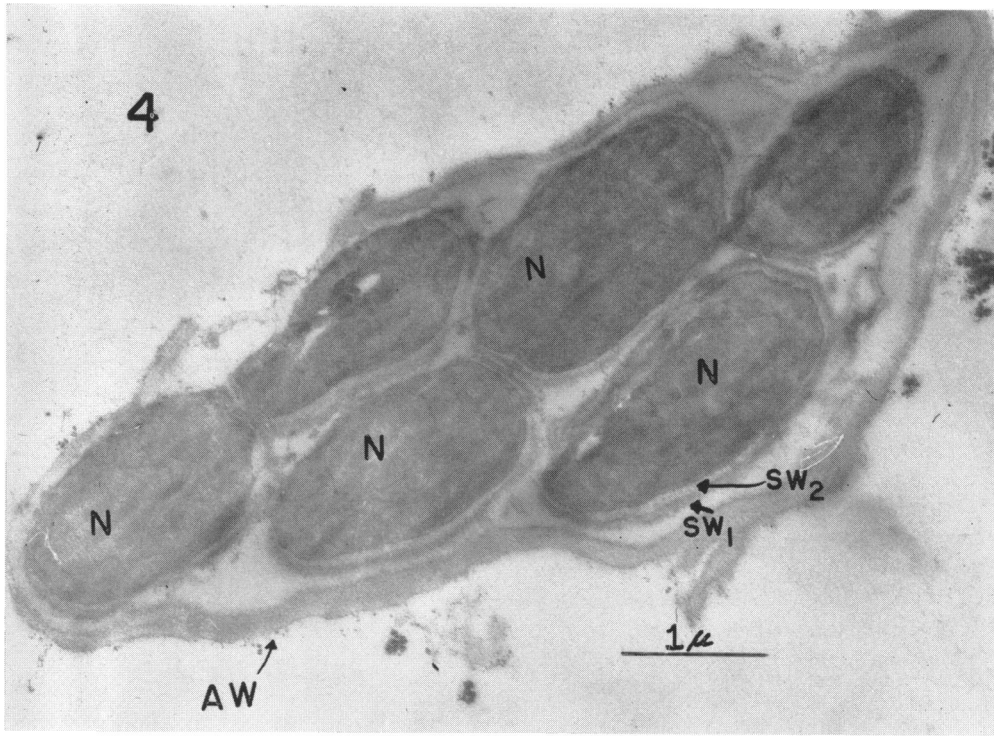


Figure 4. A section through 6 ascospores in an initial stage of germination. Note particularly the swelling and elongation of the cells and the absence of vacuoles. The outer electron-dense, and the inner spore wall of lower electron density (which gives rise to the vegetative cell wall) can also be observed.

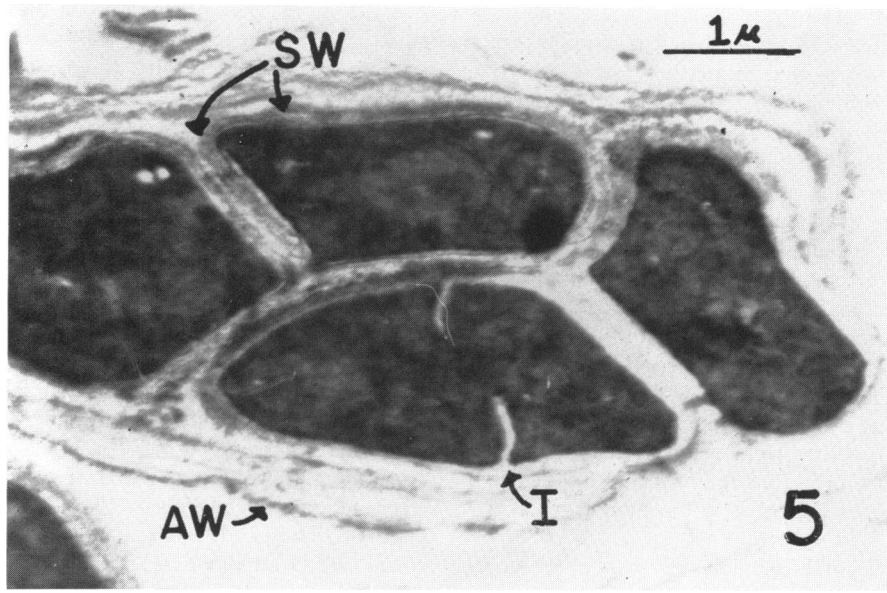


Figure 5. This micrograph of a thick section illustrates the appearance of cells in a later stage of germination. Note particularly the initiation of cell division (*I*) by inward growth of the cell wall. Note also that the ascus wall is still intact, and appears to be lamellar.

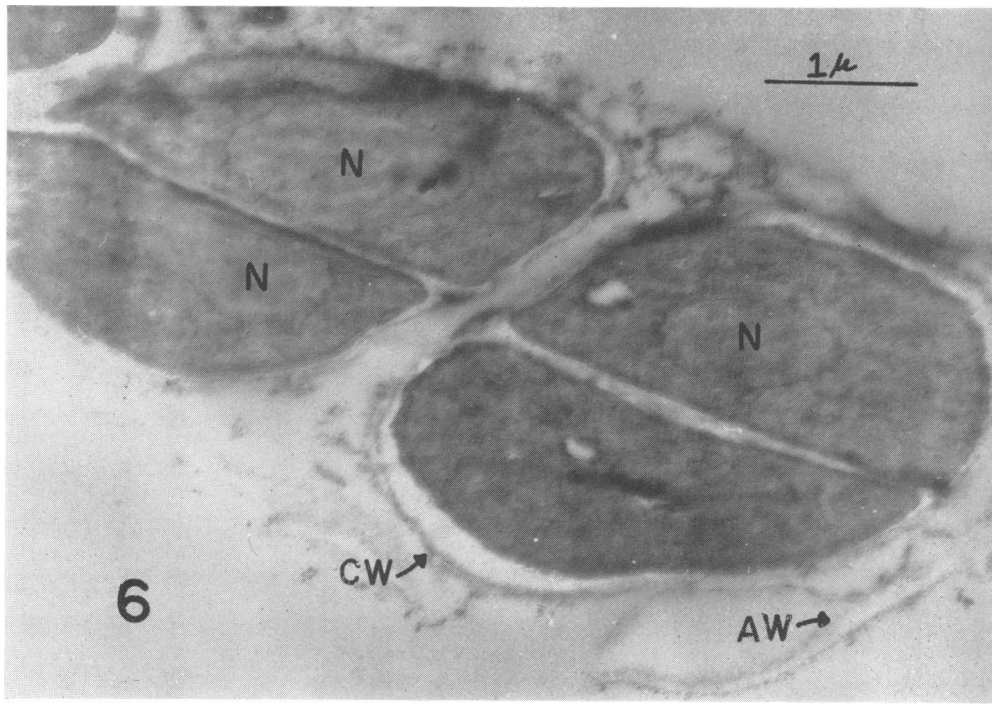


Figure 6. A micrograph of two cells which have just completed division. Note the presence and appearance of the remnants of the ascus wall (*AW*).

double membrane, although excluded from the ascospore, persist within the ascus. Observations by light microscopy reveal that these inclusions are stainable with Sudan black B. Similar structures were observed in mature asci of *S. cerevisiae* (Hashimoto *et al.*, 1958*b*). It was suggested that such structures might be remnants of mother cell mitochondria. Although mitochondria with observable *cristae* do not appear to be present in developing or mature ascospores, it may well be that some of the structures observable within the developing ascospore (*mp*, figure 1*B*) are either functional mitochondria, or structural precursors of mitochondria.

The ascus wall, like the vegetative cell wall from which it is derived, is nonelectron-dense (figure 3). During germination, however, this structure appears to develop increased density to the electron beam (figures 4 and 5). Figure 5 also indicates that it is not a monolayer structure.

The nuclei of developing and mature ascospores are conspicuously less electron-dense than the cytoplasm, and intranuclear structures were generally not observed. Examination of figure 1 however illustrates the presence of a structure which appears to be connected to the nuclear membrane of an immature ascospore. Careful observation of this micrograph indicates that only the outer membrane of this structure appears to be associated with the nuclear membrane. The inner portion of this structure appears to be comprised of a series of concentric membranes, however the resolution of the micrograph is not sufficient to make detailed observations possible.

Ascospore structure. The mature ascospore (figure 2) is surrounded by a thick, nonelectron-dense structure, the ascospore wall. Lipoidal inclusions may or may not be present. The nucleus appears to be less electron-dense than the cytoplasm and is surrounded by a double limiting membrane. Discontinuities in the nuclear membrane may correspond to the nuclear pores observed in the nuclei of vegetative cells of *S. cerevisiae* (Agar and Douglas, 1957), *Coccidioides immitis* (O'Hern and Henry, 1956) and in various animal cells (Kautz and DeMarsh, 1955; Watson, 1955). The "pores" of the ascospore nuclei may however be artifacts produced during specimen preparation. The nuclei appear to be lacking internal differentiation. Although cytostructures

with observable *cristae* were not detected, inclusions with spherical or elliptical profiles, and surrounded by a double membrane, are regularly observed in the mature ascospores.

Germination. Figure 4 illustrates that the first stage of germination consists of the swelling and elongation of the ascospores. Note particularly that the ascus wall now appears electron-dense, and that the germinating ascospores appear to be surrounded by 2 coats. The outer one appears quite electron-dense, whereas the inner one is of lower electron density. Partitioning of the cells then occurs as previously described (Conti and Naylor, 1959), the outer spore coat apparently being shed in the process. The inner spore coat therefore appears to serve as the cell wall of the vegetative cell. Figures 5 and 6 illustrate that cell division can be initiated and completed before complete disruption of the ascus wall. Cells in the later stages of germination could be differentiated in electron micrographs from vegetative cells only by the appearance of the remnants of the ascus wall around these cells. The absence of a vacuole from mature and germinating ascospores and vegetative cells lends further support to the contention that the vacuole and its contents are not an integral part of the yeast nucleus.

DISCUSSION

Previous reports from this laboratory revealed details of the cytological changes which occur during the process of germination in *S. cerevisiae* (Hashimoto *et al.*, 1958*b*), as well as information on ascospore ultrastructure. The present study clearly reveals that the structure of *S. cerevisiae* and *S. octosporus* ascospores is quite similar. Observations by both light and electron microscopy revealed no observable structural differences. Similarities in the mode of germination of the *Saccharomyces* and *Schizosaccharomyces* are also apparent. When the ascospores are put into a germination medium, swelling occurs, and the electron-dense outer spore coat is disrupted. From this time on the cells are identical with, and indistinguishable from mature vegetative cells. Similarly, the inner ascospore coat of *S. octosporus*, like that of *S. cerevisiae* (Hashimoto *et al.*, 1958*b*) gives rise to the vegetative cell wall during the germination process. This observation lends further support to the contention of Skinner *et al.* (1951), and Hashimoto *et al.* (1958*b*) that the vegetative cell wall is derived from the preexisting inner ascospore coat. It is interesting to note that observations on the germination process of bacterial endospores (Chapman and Zworykin, 1957; Mayall and Robinow, 1957) indicate that the vegetative cell wall of bacteria is formed from endospores in a similar fashion.

Previous studies of sporogenesis in *S. cerevisiae* (Hashimoto and Gerhardt, 1959) indicate that ascospore formation is initiated by the formation *in situ* of a membrane around each nucleus. This membrane is the structural precursor of the cytoplasmic membrane. The outer ascospore coat, which is lamellar in nature, is formed around the cytoplasmic membrane. The inner coat then begins to develop in the area between the cytoplasmic membrane and the outer spore coat. Subsequent increase in the thickness of this coat then occurs giving rise to structurally mature ascospores. The process of ascospore formation in *S. octosporus* appears however to be initiated by the formation of a double membrane around each nucleus. The inner portion of this membrane apparently gives rise to the cytoplasmic membrane. The ascospore wall is then formed. Although the ascospore wall of *S. octosporus* may be formed in a manner similar to that described for *S. cerevisiae*, this could not be definitely established.

Hashimoto and Gerhardt (1959) also observed and described the formation and behavior of nonelectron-dense, intranuclear structures during the sporulation process in *S. cerevisiae*. It was suggested that these structures might correspond to the chromosomes observed in the yeast nucleus by numerous workers after nuclear staining. Similar structures were not observed in electron micrographs of nuclei of sporulating cells of *S. octosporus*. The observations on ascosporogenesis in *S. cerevisiae* by Hashimoto and Gerhardt (1959) have been confirmed in this laboratory (Conti and Naylor, *unpublished observations*).

Indications of the temporary connection of the outer nuclear membrane with the internal membrane system of the cytoplasm during sporulation is not surprising in view of similar observations on budding yeast phase cells of Histoplasma (Edwards *et al.*, 1959), and sporulating cells of *S. cerevisiae* (Hashimoto and Gerhardt, 1959). The significance of these observations is unknown at present.

The structure which appears to be associated with the nuclear membrane (figure 1) may be an

artifact; however, it may be interpreted as corresponding to the central granule or centriole observed by Widra and DeLamater (1955). An intranuclear inclusion, similar in structure, was also observed in the nucleus of a budding cell of *S. cerevisiae* (figure 1, Hashimoto *et al.*, 1959). Although these "structures" may also be interpreted as being nucleoli, more definitive information must be obtained before such statements can be considered as more than mere speculation.

Previous studies (Hashimoto *et al.*, 1959) clearly indicate that the central vacuole of *S. cerevisiae* is not an integral part of the nucleus. Similar studies of *S. octosporus* (Widra and DeLamater, 1955; Conti and Naylor, 1959, 1960) resulted in a similar conclusion. The exclusion of vacuoles from the ascospore, its behavior during conjugation, and its absence from actively dividing vegetative cells of *S. octosporus* clearly indicate that the vacuolar contents are ergastic in nature, and that the vacuole is not an integral part of the nucleus. The major criterion for the belief that the yeast vacuole is an integral part of the nucleus is the presence of an intravacuolar nucleolus (C. C. Lindegren, *personal communication*). The evidence for the presence of this structure within the vacuole appears to be quite inadequate.

The results of the present study generally support and extend considerably the previous observations of sporulation and germination in *S. octosporus* by Gulliermond (1901, 1920).

SUMMARY

Sporulation, ascospore structure, and germination in *Schizosaccharomyces octosporus* were studied by means of ultrathin sectioning and electron microscopy. Sporulation is initiated by the formation of a double ascospore membrane around each nucleus. The inner portion of this membrane appears to serve as the structural precursor of the cytoplasmic membrane. The ascospore wall then develops around the ascospore membrane and gradually increases in thickness. Mature and germinating ascospores are surrounded by 2 spore coats, the inner one giving rise to the vegetative cell wall. Germination is initiated by swelling and elongation of the cells with subsequent disruption of the outer spore coat. At this stage vegetative cell division is initiated. Further evidence is presented to support the contention that the yeast vacuole is not an integral portion of the nucleus. Ascosporogenesis, ascospore structure, and germination of *Saccharomyces cerevisiae* and *S. octosporus* are compared.

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