ELECTRON MICROSCOPY OF ULTRATHIN SECTIONS OF *SCHIZOSACCHAROMYCES OCTOSPORUS*

II. Morphological and Cytological Changes Preceding Ascospore Formation

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Conjugation (plasmogamy) in yeast was first described by Shionning (1895). He observed that ascus formation in *Schizosaccharomyces octosporus* was preceded by fusion of two sister cells. Further studies by Hoffmeister (1900) indicated that nuclear fusion accompanied cell fusion. Guilliermond (1901) clearly demonstrated that nuclear fusion occurred after cell fusion, and that ascus formation is preceded by a true isogamous conjugation. Further studies of sporogenesis, utilizing the iron-hematoxylin techniques were carried out by Guilliermond (1917). Recent studies by Widra and DeLamater (1955), describe the occurrence of a typical meiotic cycle in *S. octosporus* after the haploid cells have united. Lindegren (1951) described the involvement of the vacuole during copulation of *Saccharomyces bayanus*, whereas Widra and DeLamater (1955) tend to confirm Guilliermond's belief that the vacuolar contents are ergastic in nature.

Although general agreement appears to be lacking, these various studies revealed the sequence, and nature of some of the cytological changes which occur during plasmogamy, karyogamy, and subsequent nuclear division. The process of conjugation in *S. octosporus* was therefore studied by electron microscopy in the hope that the observations of other investigators by light microscopy could be correlated with our observations by electron microscopy, thereby leading to a more refined and accurate description of the cytological changes which precede ascospore formation in *S. octosporus*.

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MATERIALS AND METHODS

The culture used in these studies was *S. octosporus* strain NRRL Y-854, 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, and 1.5 per cent agar added when a solid medium was required. Conjugating cells were obtained by inoculating 24-hr-old cultures onto YM agar plates (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. Cells were collected at appropriate time intervals for study by electron microscopy. Sporulation was essentially complete within 48 to 72 hr.

Specimen preparation. Cells were collected from agar surfaces by washing off with 0.06 M phosphate buffer at pH 6.6. Cells were then sedimented by centrifugation and resuspended in a 1.5 per cent aqueous solution of $KMnO_4$ for 40 min at 4 C. All subsequent steps, except for the polymerization were also carried out at 4 C. Specimens were dehydrated by serial passage (1 hr each) into 70, 95, and 100 per cent ethanol, transferred to a 50 per cent solution of butyl methacrylate in absolute alcohol, and left for 1 to 2 hr. Cells were then passed twice (½ hr each) through cold butyl methacrylate, and transferred to partially polymerized butyl methacrylate. Specimens were left in the latter solutions for 6 to 8 hr, transferred to gelatin capsules, and allowed to settle for 4 hr at room temperature. Polymerization was accomplished by placing the capsules in an oven at 56 to 60 C for 8 to 12 hr. Ultrathin sections were obtained by means of a Porter-Blum ultramicrotome equipped with a glass knife. Difficulty in sectioning due to the softening of the block could be overcome by

leaving the blocks at 4 C before sectioning. Sectioning could also be facilitated by exposing the tip of the block to a stream of liquid nitrogen for 20 to 30 sec, approximately 20 min before sectioning. Condensed water was removed by carefully blotting with filter paper. The technique of Satir and Peachey (1958) was employed to decrease decompression artifacts. Sections of less than 0.1 μ were picked up on 200 mesh copper grids on which a collodion, Formvar, or carbon film had been mounted. Sections were examined in an RCA-EMU 2B electron microscope equipped with a 50 or 100 μ objective aperture.

Cytochemical techniques and light microscopy. Several cytochemical methods were used to correlate structures observed in electron micrographs with structures observable by light microscopy. Neotetrazolium salts and dilute Lugol's solution were used, respectively, for the demonstration of mitochondria and glycogen. Lipoidal inclusions were identified by their stainability with Sudan black B. Copulation was also followed by means of phase microscopy to confirm the general appearance of the cells during the sequence of events leading to ascus formation. The shape of the cells and previous studies by phase and electron microscopy (Conti and Naylor, 1959*a*) of dividing cells of *S. octosporus* made it possible to clearly distinguish between dividing and conjugating cells. Since vacuoles are absent from actively dividing cells of *S. octosporus* (Ganesan and Swaminathan, 1958; Conti and Naylor, 1959*b*) and present in conjugating cells, the development and fate of this structure was of particular interest.

RESULTS

Light microscopy. Observations by light microscopy confirmed many of the previous observations of the conjugation process (Guilliermond,

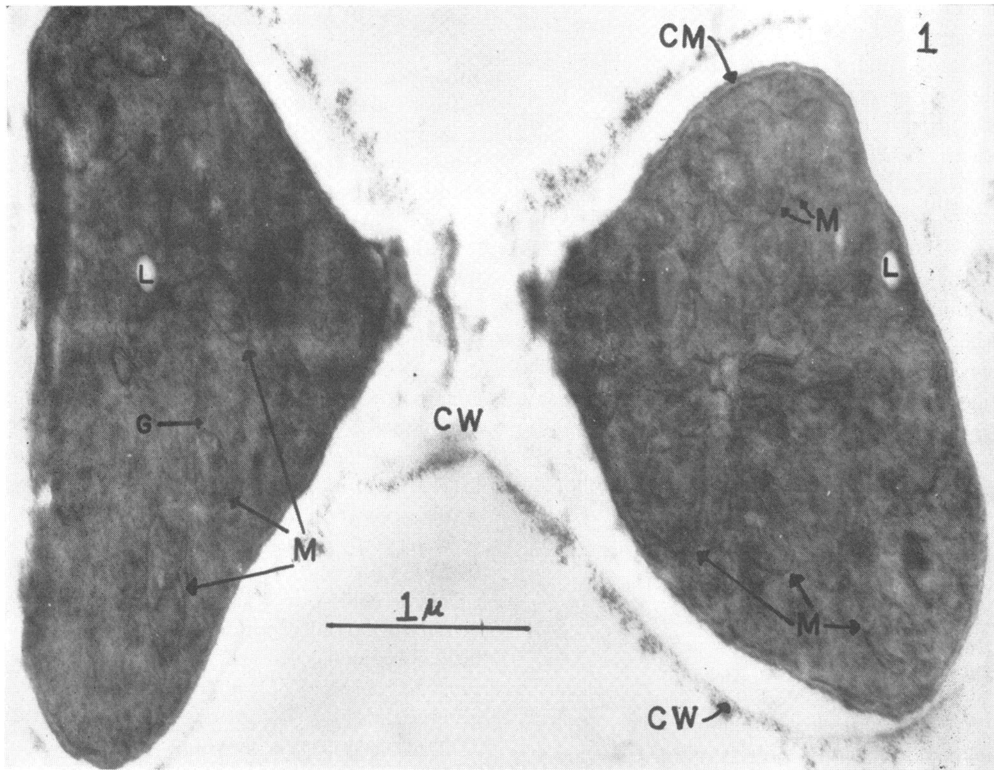
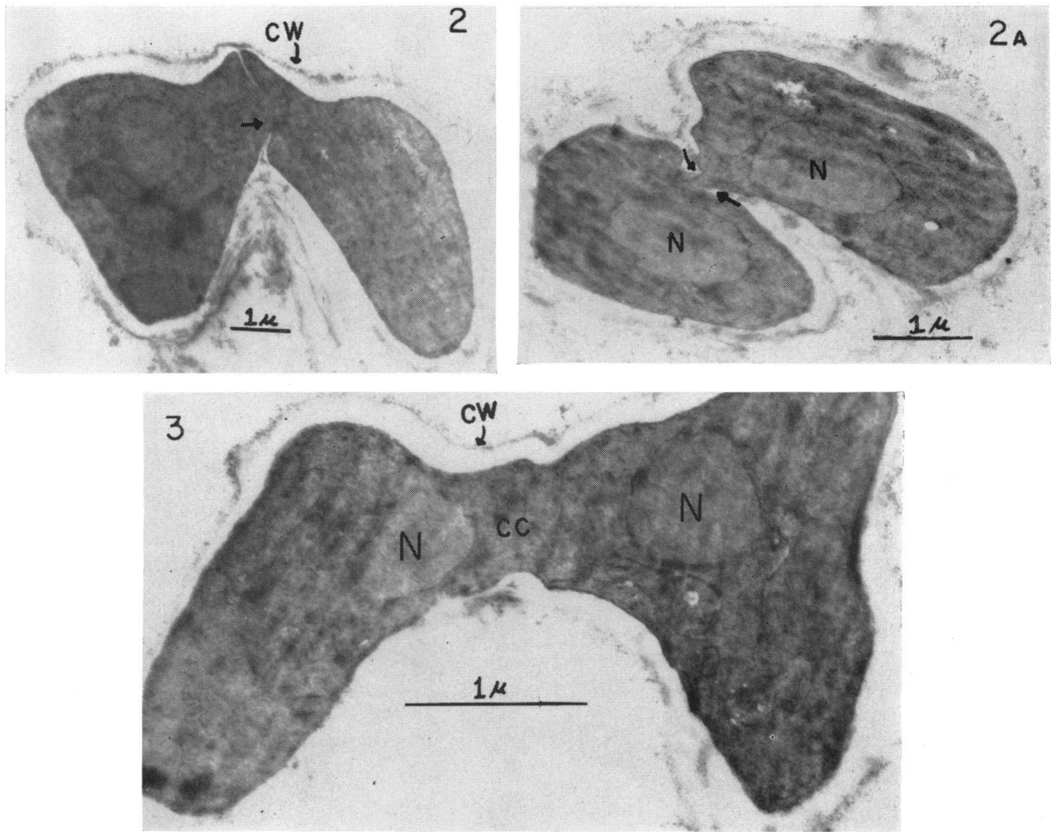


Figure 1. An oblique section of two cells during an initial stage of conjugation. Structures labeled M are double membrane chambers containing *cristae*. Note particularly the prominent cytoplasmic membrane, and the partial fusion of the cell walls. Small, unidentified, electron-dense inclusions (*G*) can also be observed. Few lipoidal inclusions are observable, and vacuoles appear to be absent.



Figures 2 and 2a. Figure 2 is a micrograph of two cells immediately prior to cytoplasmic fusion. The cytoplasmic membranes (at site of arrow) are thin, distinct, and still intact. Figure 2a illustrates that dissolution of the central portion of the connecting cell wall occurs, thereby leading to cytoplasmic fusion (arrows point to area of cytoplasmic intermixing).

Figure 3. This micrograph illustrates the increase in length and width of the conjugation canal causing the ascus to appear halter-shaped. Note that the nuclei appear to be migrating towards the canal.

1920; Widra and DeLamater, 1955). The conjugation process appears to be initiated by the attachment of the cells in pairs by a portion of the cell wall. The area of contact then increases, and the cells appear to be firmly joined by a common cell wall. The central portion of the connecting cell wall then disintegrates, thereby forming a short, narrow conjugation canal. This canal subsequently increases both in length and width, giving the ascus a dumbbell appearance. Further increase in the width of the conjugation canal gives the ascus a rectangular shape. Ascospore formation appears to occur after the ascus has assumed a rectangular appearance. It appeared, contrary to the observations of Guilliermond (1920), that the copulation canal is not formed by the fusion of short projections from each cell.

Examination of actively dividing vegetative cells of *S. octosporus* by means of phase microscopy and staining with dilute Lugol's solution and Sudan black B revealed that a vacuole was not present, and lipoidal inclusions lacking or few in number (Conti and Naylor, 1959*a*). Similar studies of conjugating cells revealed that vacuoles could be clearly observed only in cells joined by a well developed conjugation canal. There was no increase in the number of inclusions stainable with Sudan black B. Tetrazolium salts staining of actively dividing vegetative cells, and of copulating cells revealed a noticeable increase in the number of stainable bodies (mitochondria) during the initial stages of copulation.

Electron microscopy. Figures 1 to 10 are electron micrographs of *S. octosporus* illustrating suc-

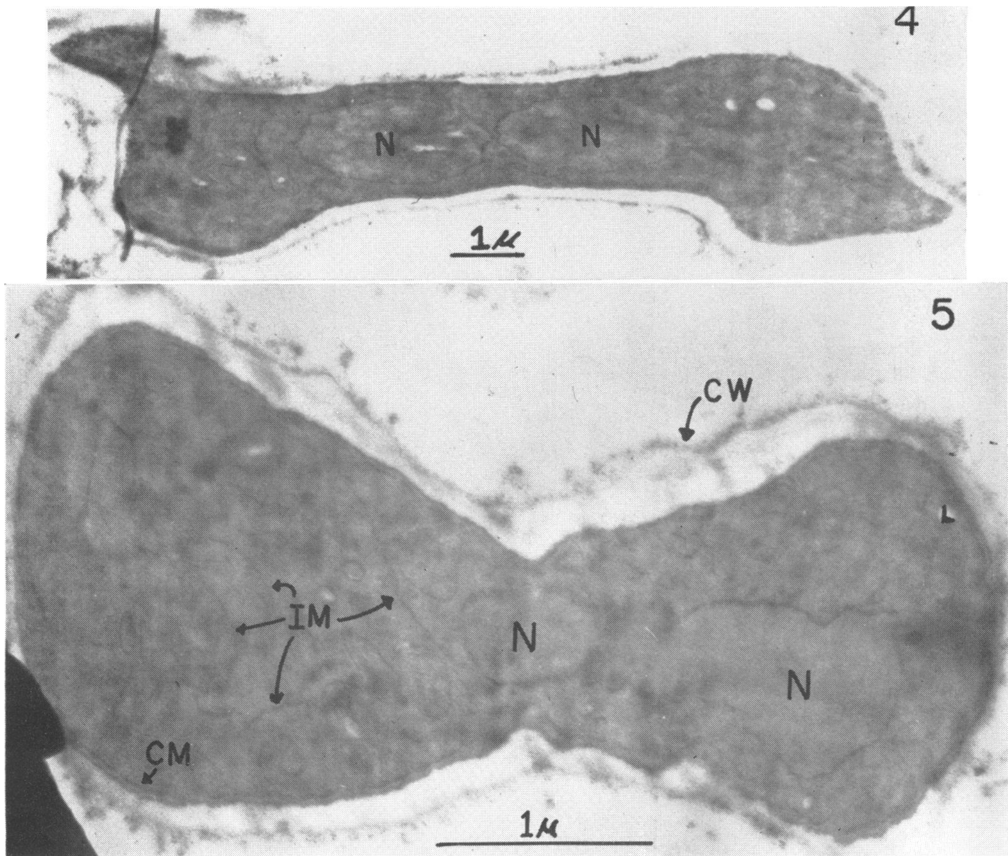


Figure 4. Migration of the two nuclei towards one another, immediately prior to fusion

Figure 5. Nuclear fusion. Note that the nuclear membrane remains intact except at the site of fusion. The presence of an internal membrane system is also apparent.

cessive stages of copulation and nuclear division. The sequence of events is based on the preliminary studies by phase microscopy, and previous reports of other investigators (Guilliermond, 1920; Widra and DeLamater, 1955). The shape and size of the growing ascus was useful in reconstructing the successive stages from electron micrographs. Widra and DeLamater (1955) also used the shape and size of the ascus as an independent indicator of timing and reported that there was a correlation between nuclear development and the appearance of the ascus.

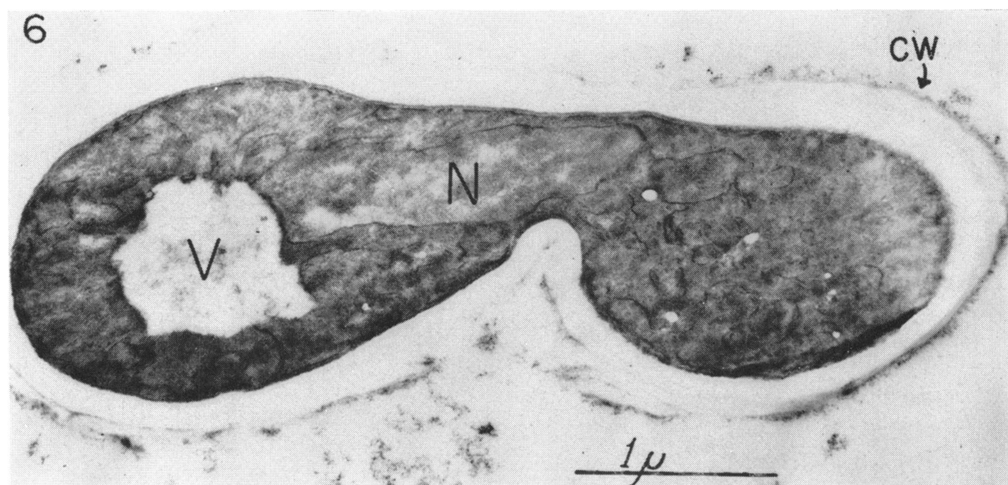
Nuclei are regularly observed in sections of conjugating cells during both initial and later stages of the conjugation process. The structure labeled *N*[3] in the micrographs is considered to be the nucleus on the basis of its behavior during cell division (Conti and Naylor, 1959*a*) and the occurrence and behavior of a similar structure in dividing (Hashimoto *et al.*, 1959), sporulating (Hashimoto and Gerhardt, 1959), and germinating cells (Hashimoto *et al.*, 1958) of *Saccharomyces cerevisiae*. The nuclei in conjugating cells appear to be similar in structure to the nuclei of dividing cells (Conti and Naylor, 1959*a*). Nuclei are surrounded by a double membrane, are ovoid to spherical in shape, and the nucleoplasm appears to be granular and homogeneous in texture.

The ascus wall, like that of the vegetative cell wall, was relatively wide, and appeared to be of low electron density in electron micrographs. The cytoplasmic membrane appeared to be double, and closely adherent to the ascus wall. Spherical or elliptical inclusions, which appear to be empty in the micrographs, are similar in size, shape,

[3] *CC* = conjugation canal; *CM* = cytoplasmic membrane; *CW* = cell wall; *IM* = internal membranes; *L* = lipoidal inclusion; *M* = mitochondrion; *N* = nucleus; and, *V* = vacuole.

and number to the structures stainable with Sudan black B.

Figure 1 illustrates the appearance of two vegetative cells during the initial stages of copulation. It can be seen that the cell walls are at least partially fused, with no indications of the prior formation of projection tubes. Nuclei cannot be observed due to the plane of sectioning. The absence of nuclei facilitates observations of other cytostructures within these cells. In agreement with the results from the light microscopy studies, vacuoles were not present, and lipoidal inclusions absent or few in number. Structures having circular or ellipitical profiles, and pos-



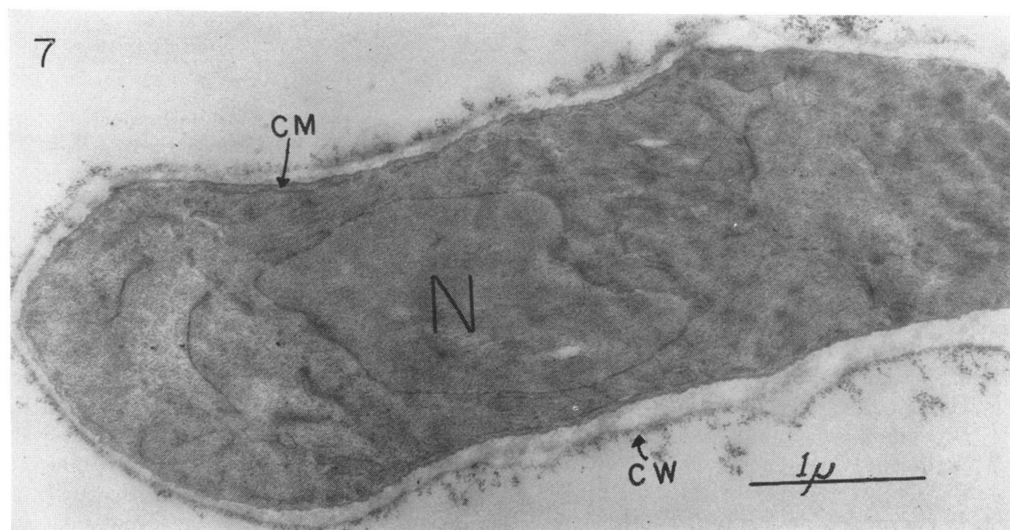


Figure 6. This figure illustrates the dumbbell appearance of the ascus, and the size of the nucleus subsequent to nuclear fusion. Note that the fusion nucleus (approximately twice the size of the vegetative nucleus) spans almost one-half of the length of the ascus. A conspicuous vacuole can be readily observed. Note also the low-electron dense, and continuous ascus wall.

Figure 7. A cross section of a mature ascus, i.e., an ascus in which the conjugation canal is no longer evident. At this stage, immediately prior to nuclear division, the nucleus appears to be contracted, and assumes an irregular shape. Note again the conspicuous cytoplasmic membrane.

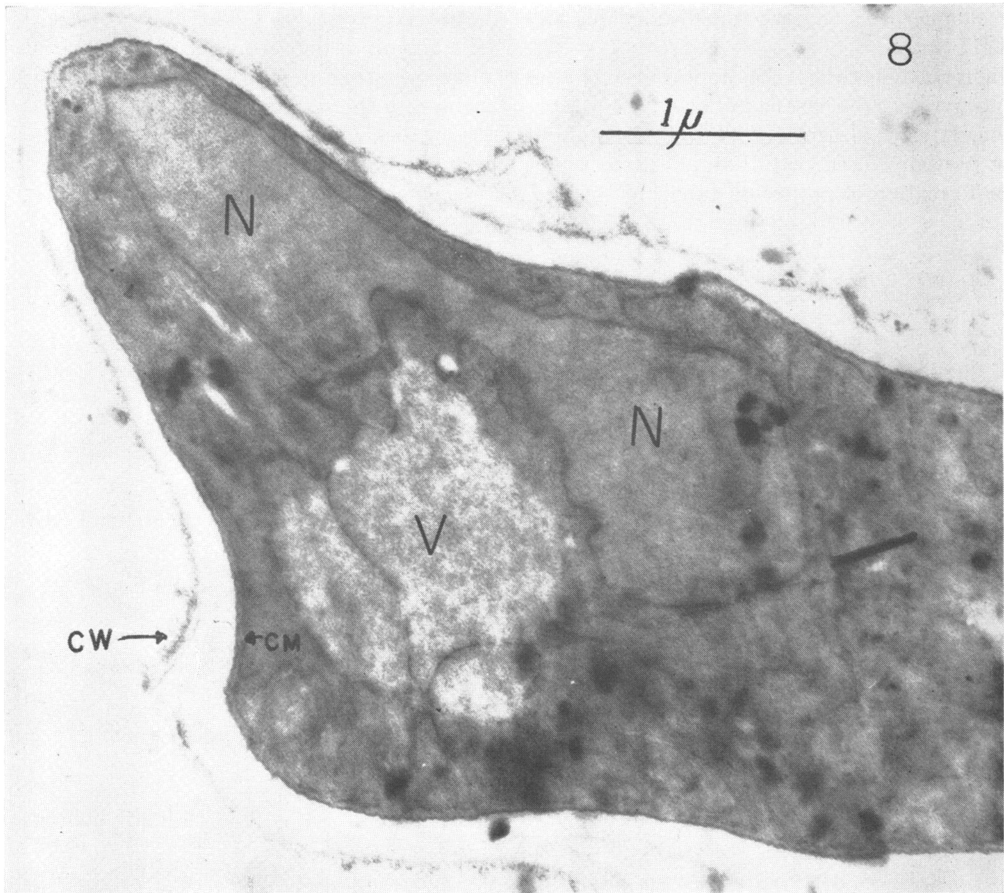


Figure 8. This particularly informative micrograph illustrates the behavior of the nucleus during the first division. The nucleus appears to elongate, and divide by a process of constriction. Note that the vacuole is separated from the dividing nucleus, and appears to contain granular or amorphous material. It is evident that the nuclear membrane remains intact.

sessing an internal membrane system, can be observed in the cytoplasm of the conjugating cells. These structures are considered to be mitochondria on the basis of their ultra structure in electron micrographs, and the number and size of similar structures in conjugating cells stained with tetrazolium salts and observed with the light microscope. These mitochondria are randomly distributed throughout the cell, and appear to be similar in structure to the mitochondria of other yeast cells (Agar and Douglas 1957; Hashimoto *et al.*, 1958, 1959). Previous studies (Conti and Naylor, 1959*a*) revealed that the mitochondrialike structures were few in number in dividing vegetative cells, and *cristae* difficult to observe. It thus appears that during the initial stages of copulation, cellular reorganization includes an increase in number of mitochondria, and an alteration of mitochondrial structure.

Subsequent to cell wall fusion, the central portion of the connecting cell wall appears to disintegrate (figures 2 and 2*A*), resulting in a cytoplasmic intermixing. The copulation canal then appears to increase in both length and width, giving the ascus a characteristic appearance (figure 3). At this stage of the process the nuclei then begin to migrate towards the copulation canal (figure 4). Nuclear fusion then occurs (figure 5). It is interesting to note that the limiting membranes of the fusing nuclei remain intact, except in the region where fusion occurs. Nuclear fusion was always found to occur within the conjugation canal. It is pertinent to mention at this point that vacuoles do not appear to be present in the

ascus until nuclear fusion has occurred (figures 1–6). The preliminary studies employing iodine staining, and observations by phase microscopy, also indicated that vacuoles were formed in the majority of cells during, or immediately after, fusion nucleus formation. The vacuole, when present, usually appeared to contain granular or amorphous material.

Figure 6 illustrates the appearance of the ascus after the fusion of the nuclei. Note the characteristic dumbbell appearance of the ascus and the conspicuous vacuole. The fusion nucleus (presumably diploid) appears to be elongate in shape, and spans approximately half the length of the ascus. Calculations from electron micrographs of the volume of the nucleus at this stage reveal that the fusion nucleus is approximately twice that of the vegetative cell nucleus.

The fusion nucleus, soon after its formation, often appears to assume an irregular shape (figure 7). Presumably, this stage precedes division of the nucleus by the process of constriction illustrated in figure 8. This figure is particularly informative. It clearly demonstrates that the vacuole is separated from the dividing nucleus, and that the nuclear membrane remains intact during the first division of the nucleus. The process of nuclear division can best be described as a constriction within the central region of the nucleus, yielding two nuclei of equal size, and presumably also equal in content.

The two nuclei then migrate to opposite ends of the ascus (figures 9*A* and 9*B*). At this stage the nuclei again appear to begin to constrict and separate, thereby resulting in the formation of four nuclei. Note particularly the size of the

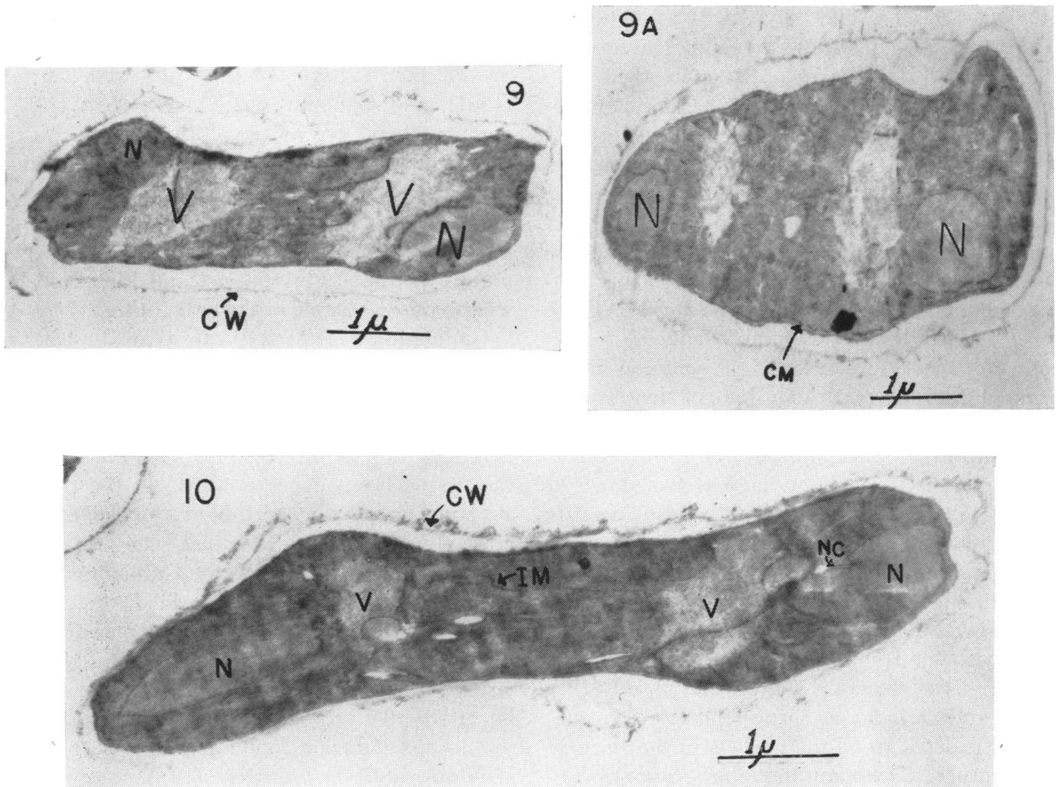


Figure 9 and 9a. These micrographs illustrate that after the first division, the nuclei migrate to opposite ends of the ascus. Note that the nuclear membrane is still intact, and that these nuclei appear to be approximately the same size as vegetative cell nuclei.

Figure 10. The size of the nuclei, shape of the ascus, and the observable constriction (*NC*) of one of the nuclei indicates that the second nuclear division is occurring. Note again that the nuclear membranes are still intact. The presence of the internal membrane system and low-electron dense ascus wall are still apparent.

nuclei in figure 10, and the invagination of the nuclear membrane of one of the nuclei, indicating that constriction of the nucleus is occurring. Unfortunately, electron micrographs which could clearly illustrate the subsequent stages of nuclear division were not obtained. Some electron micrographs indicated that nuclear division continued in the manner described, resulting in the formation of eight nuclei, each being surrounded by a limiting membrane. The interpretation of these micrographs is, however, difficult due to a variety of reasons, and therefore subject to criticism. Attempts are now being made to obtain complete serial sections of a single cell in order to overcome difficulties in interpretation.

DISCUSSION

The observations made by means of electron microscopy are generally in agreement with the previous observations by light microscopy (Guilliermond, 1920; Widra and DeLamater, 1955; Yoneyama, 1958). Although there are several points of disagreement, the previous observations by light microscopy and the present studies by electron microscopy clearly supplement each other.

Widra and DeLamater (1955) utilizing refined nuclear staining techniques were able to observe the behavior of the nucleus during conjugation and sporogenesis of *S. octosporus*. These investigators, however, found it difficult to discern the presence or absence of a nuclear membrane due to the resolution limit of their optical system. The present studies by electron microscopy, however, illustrate that the nuclear membrane persists throughout the first two stages of nuclear division, and probably also persists throughout the third division. The observations of Widra and DeLamater on the size, location and behavior of the nucleus, particularly during early stages of conjugation, are in close agreement with the results of the present study. It is clear that the structure stained and observed by Widra and DeLamater corresponds to the structure considered to be the nucleus in the electron micrographs. These authors, however, also observed chromosomes within the nucleus, and reported the occurrence of a classical meiotic process during nuclear division. Chromosomes could not be observed in the nucleus utilizing the techniques employed in the present study. The difficulties involved in observing intranuclear structure particularly chromosomes, by means of ultrathin sectioning and electron microscopy are briefly discussed by Cosslett (1958) and Conti and Naylor (1959*a*). It is apparent, however, that despite the limitations of both light and electron microscopy, observations by both techniques can be closely correlated. It is also apparent that investigation of structure and behavior of chromatin material within the yeast nucleus by electron microscopy must await further development and refinement of techniques. Application of methods in this laboratory, similar to those employed by Moses (1956), to a study of the yeast nucleus have not been successful to date. Until further techniques are developed the description of structure and behavior of the intranuclear material of the yeast cell remains primarily in the realm of light microscopy. Present studies in this laboratory utilizing various nuclear staining techniques indicate the presence of chromosomelike structures within the membranes of ascus nuclei of *S. octosporus*, however classical meiotic figures have not been observed. Cutter (1951), and Ganesan and Swaminathan (1958) among others, are aware of the deficiencies of the techniques of light microscopy that are employed to study the fungal and yeast nucleus and cite the need for the development of more refined procedures. It is hoped that newer techniques for the study of yeast and fungal nuclei will become available in the near future.

Guilliermond (1920) indicates that conjugation is initiated by the formation and fusion of projections from each cell. Although the appearance of conjugating cells under the light microscope may give this impression, this apparently is not the case. The evidence obtained in the present study by employing both light and electron microscopy strongly indicates that such projections are not formed. The views of Guilliermond are probably due to the difficulty in distinguishing between cell wall material and cytoplasm in unstained yeast cells examined with the light microscope. The conflicting observations also may be attributed to differences in strains and methods of inducing copulation and sporulation.

Widra and DeLamater (1955) state that "... two vegetative haploid cells unite by means of fusion tubes to form an ascus." Evaluation of their evidence for this statement is difficult since their micrographs are poorly reproduced, and the figures referred to are of cells which have been subjected to nuclear staining. The use of the term "fusion tubes" is also unfortunate since

these authors may be referring to the copulation canal. It is recommended that the term "fusion tube" not be used in the description of the early stages of copulation in yeast.

The process of copulation in the yeast cell has been described by Lindegren (1951). The behavior and identification of various structures, and particularly the involvement of the vacuole in the conjugation process, are not confirmed. The evidence from the present study supports the view of Widra and DeLamater (1955) and Guilliermond (1920) that the vacuolar contents are ergastic in nature and that the vacuole is not an integral part of the yeast nucleus. The structure and behavior of the organelle considered to be the centrosome by Lindegren, seems to correspond to the structure considered to be the nucleus in this study.

These studies shed no light on the question of the presence of an intranuclear nucleolus in yeast (Caspersson and Brandt, 1941; Lietz, 1951; Widra and DeLamater, 1955). A structure corresponding to the centriole also was not observed in any electron micrographs. The failure to observe these structures in electron micrographs does not establish or indicate that these structures are not present. The inability to observe such structures may be ascribed to a variety of factors (Moses, 1956; Cosslett, 1958; Conti and Naylor, 1959*a*).

The cytological changes accompanying ascospore formation ("free cell formation") and germination of ascospores are currently under investigation.

SUMMARY

Electron micrographs illustrate the cytological changes occurring during plasmogamy, karyogamy, and subsequent nuclear division in *Schizosaccharomyces octosporus*. The conjugation process appears to be initiated by the attachment of the cells in pairs by a portion of the cell wall. Fusion of cells by means of projection tubes was not observed. The central portion of the connecting cell wall then disintegrates, thereby forming a short, narrow conjugation canal which subsequently increases both in length and width. At this stage, the nuclei migrate toward one another, and fuse within the canal. The limiting membranes remain intact except in the region of nuclear fusion. Subsequent nuclear division appears to occur by a process of constriction. Intranuclear structures were not observed.

The structure and behavior of other components of the yeast cell during the conjugation process are also described. Observations on the vacuole lend further support to the view that the vacuole is not an integral part of the nucleus.

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