FINE STRUCTURE OF MICROORGANISMS

III. Electron Microscopy of Resting and Germinating Ascospores of *Saccharomyces cerevisiae*[1]

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Although there is a voluminous literature concerning the structure and function of various organelles within the yeast cell, general agreement seems to be lacking. Various controversies and disagreements have arisen, due primarily to differing interpretations of light microscopical observations. Many studies have been carried out on the behavior and structure of these organelles during various phases of development of the yeast cell; however, no detailed studies have been carried out on the internal structure of ascospores of *Saccharomyces cerevisiae*. A study by ultrathin sectioning and electron microscopy by Agar and Douglas (1957) of resting vegetative cells of *S. cerevisiae* increased the possibility of resolving some of the disputes apparently caused by the limited resolution of the light microscope. Although their technique was successful for study of the vegetative yeast cell when grown on Lindegren's presporulation medium, detailed observations on the internal structure of the yeast ascospore could not be made due to lack of internal differentiation and to disruption during the preparative process. In the present investigation, suitable methods were devised which made it possible to observe the internal structure of ascospores of *S. cerevisiae*. Structural changes during germination were also investigated.

MATERIALS AND METHODS

Yeast. A distillers yeast, *Saccharomyces cerevisiae* strain M-1, was employed. Cells were routinely grown on a molasses medium (molasses, 10 per cent; tryptone, 0.1 per cent; yeast extract, 0.1 per cent; ammonium sulfate, 0.1 per cent; agar, 2.5 per cent) at pH 4.8 for 3 days at 30 C. A high rate of sporulation (approximately 90 per cent) was obtained by washing the cells from the surface of the molasses agar with sterile distilled water, and inoculating heavily onto an acetate-glucose agar medium (glucose, 0.1 per cent; agar, 2.0 per cent; in 0.1 M acetic acid-sodium acetate buffer at pH 4.8). This medium is similar to the sporulation medium described by Adams (1949). Sporulation was essentially completed after incubation for 40 hr at 30 C. These spores remained dormant for at least 1 month even when kept at 30 C. Ascospores for germination studies were obtained by harvesting 3 day old cultures grown on the acetate-glucose medium, washing twice in sterile distilled water, and inoculating heavily into a germination medium at pH 6.0 (glucose, 1.0 per cent; yeast extract, 2.0 per cent; peptone, 0.5 per cent; KH_2PO_4, 0.1 per cent; $MgSO_4$, 0.05 per cent). Cultures were then incubated with vigorous shaking at room temperature. Under these conditions, germination was complete within 6 to 8 hr. Aliquots of the culture were taken at appropriate intervals for study by light and electron microscopy.

Preparation of ultrathin sections. The techniques employed were essentially the same as described by Hashimoto and Naylor (1958*a*). The major modification was the substitution of potassium permanganate for osmium tetroxide as the fixing agent. The relative value of these agents as fixatives for animal tissues has been reported by Luft (1957). Although osmium tetroxide gave superior results insofar as cell preservation was concerned, differentiation of internal structure after sectioning was far superior in the case of permanganate treated cells. Time of fixation and concentration of permanganate were extremely critical since this fixative had a narrow latitude for successful fixation of yeast cells. Excellent results were obtained when the cells were fixed with a 1.5 per cent aqueous solution of potassium

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permanganate for 30 min at room temperature. Prolonged treatment with partially polymerized *n*-butyl methacrylate minimized explosion of the cells during polymerization.

Sectioning and electron microscopy. Sections were cut with a Porter-Blum ultramicrotome equipped with a glass knife. Occasional preparations were made employing Formvar rather than collodion for coating the grids. An RCA EMU-2b electron microscope, equipped with a 50 μ limiting objective aperture was used for all electron microscope observations.

Cytological techniques and light microscopy. Various cytochemical methods were employed in an attempt to correlate structures observed in electron micrographs with structures observable with the light microscope after appropriate treatment. Lipid components were stained with Sudan black B. Neotetrazolium salts and Lugol's solution were used, respectively, for the demonstration of mitochondria and glycogen. A criterion for germination was complete loss of resistance of the spores to basic dyes after alcohol fixation.

For the purpose of comparison with the bacterial endospore, sections of both resting and germinating ascospores were treated with a warm 1 per cent aqueous solution of lanthanum nitrate (Mayall and Robinow, 1957) for various periods of time. Sections were then refloated on clean distilled water for removal of nonspecific precipitants before drying for electron microscopy.

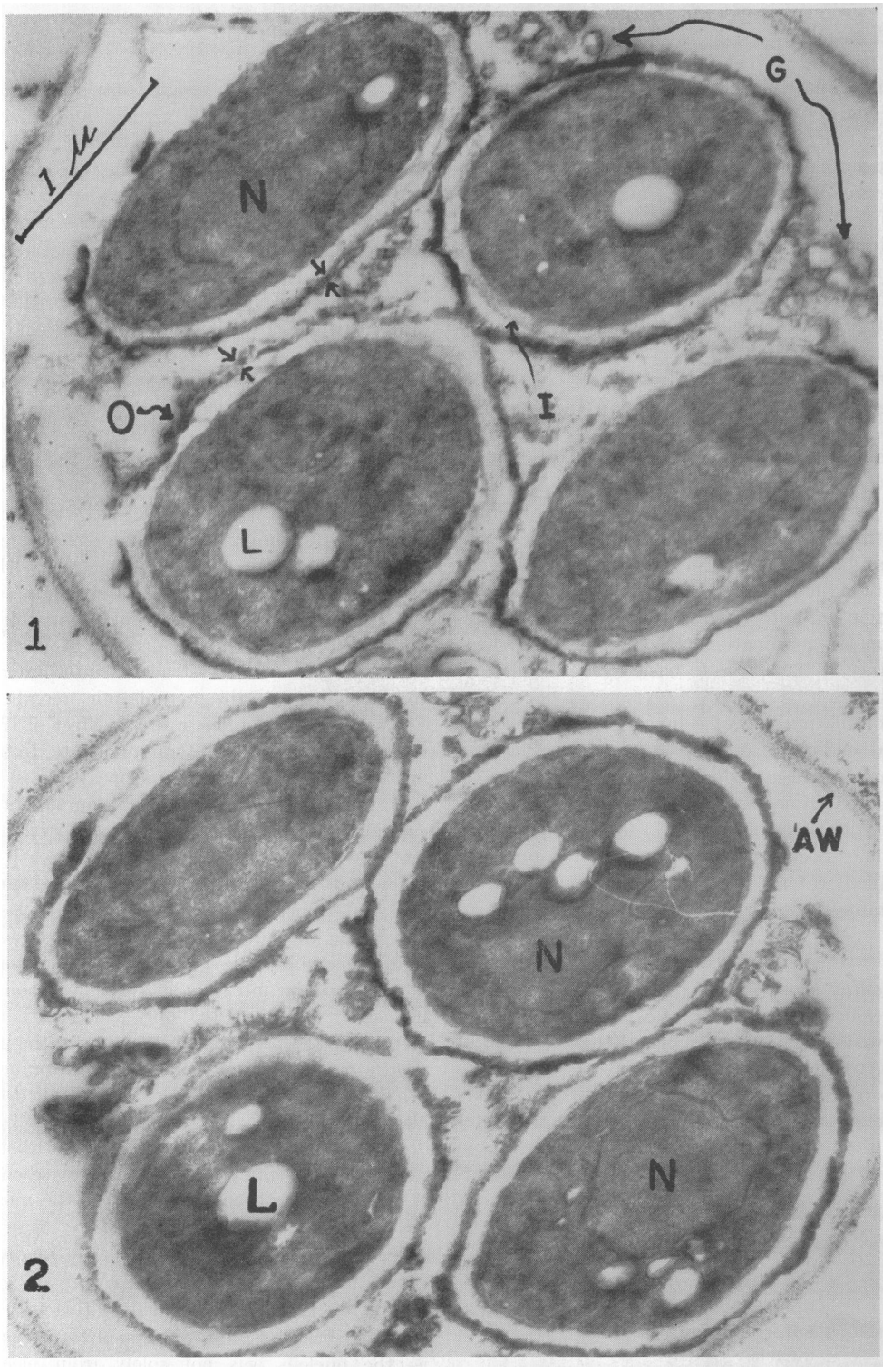
RESULTS

Resting ascospores. Figures 1 and 2 show the same dormant ascospores of *S. cerevisiae* sectioned in two different parallel planes. Examination of these electron micrographs reveals that each ascospore is surrounded by two definite, easily distinguishable layers, referred to as the outer and the inner spore coats. The outer coat (*O* in figure 1)[3] is characteristically dense to the electron beam, and has a rough outer surface. It is approximately 300 to 400 A thick, and there are indications that it is composed of more than one layer (double arrows in figure 1). In contrast to this, the inner coat appears to be much less dense to the electron beam, and has a smooth surface. Close observation of the area lying between the outer coat and the spore cytoplasm reveals the presence of this inner spore coat (*I* in figure 1) which is approximately 700 A thick. The low density of the inner coat persists throughout the germination process and in young vegetative cells. In some of the figures it may appear to be absent since demonstration requires sacrifice of the internal structure of the spores and vegetative cell in the printing of electron micrographs.

In routinely prepared sections, a definite entity corresponding to the cytoplasmic membrane could not be clearly demonstrated. However, treatment of sections with a warm 1 per cent aqueous solution of lanthanum nitrate appreciably increased the density of both the inner spore coat and a structure interpreted as corresponding to the cytoplasmic membrane. Figures 4 and 5 demonstrate the effect of treatment of sections with lanthanum nitrate. In these micrographs the inner coat appears as a uniformly thick and smooth layer completely occupying the area between the spore cytoplasm and the outer coat. Immediately beneath the inner coat a fine but distinct membrane can be observed, which we refer to as the cytoplasmic membrane. Careful observation of figure 5 reveals that the cytoplasmic membrane is invaginated at fairly regular intervals. This phenomenon can be clearly demonstrated only with lanthanum nitrate treated preparations. Except for the inner spore coat and the cytoplasmic membrane no other structures develop increased density to the electron beam.

The nucleus in resting ascospores. A distinct morphological entity with the vegetative cell of *S. cerevisiae* has been referred to as the nucleus by some workers (Hawthorne, 1955; Agar and Douglas, 1957) and as the spindle by Lindegren *et al.* (1956). A similar structure (*N*) in all figures will be referred to as the nucleus in this presentation. The basis for interpretation of this structure as the nucleus was not solely from electron micrographs or cytochemical observations, but also from observations on its structure and behavior during vegetative cell division. This latter

[3] *AW* = ascus wall
CM = cytoplasmic membrane
G = ascus granule
I = inner ascospore coat
L = lipoidal inclusion
M = mitochondrion
N = nucleus
O = outer spore coat
S = invaginations of cytoplasmic mmembrane
V = vacuole.



Figures 1 and 2. Electron micrographs of an ascus containing four ascospores sectioned in two different parallel planes. Note the advantages of serial sections for demonstrating the presence of the nucleus and other structures within the ascospores. The inner ascospore coat and lamellar nature of the outer spore coat are apparent.

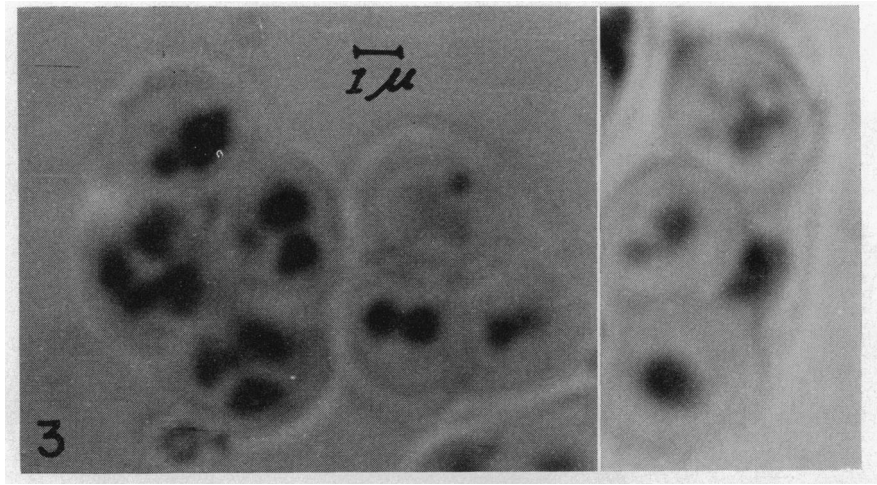


Figure 3. Light micrographs of dormant asci stained with Sudan black B. These deeply staining bodies correspond to the structure *L* in the electron micrographs.

study is to be published elsewhere. Although occasional spores appeared to be lacking in this structure, serial sections of the same spore in several different parallel planes always revealed its presence. In no case was there ever found to be more than one nucleus in an individual spore. The nucleus appeared to be lobulate and was enclosed within a double membrane. The nuclear pores observed in various animal cells (Rhodin, 1954; Kautz and DeMarsh, 1955; Watson, 1955) in *Coccidioides immitis* (O'Hern and Henry, 1956) and in the vegetative yeast cell (Agar and Douglas, 1957) could not be convincingly demonstrated, although close inspection of the nuclear membrane revealed areas which might be interpreted as being pores. The nucleus appeared to be devoid of internal differentiation, and was found to be less electron dense than the cytoplasm. The nucleus of the ascospore is similar in structure to the nucleus of the resting vegetative cell (Agar and Douglas, 1957).

Cytoplasmic inclusions. Observations of the electron micrographs revealed that several spherical vacuoles were usually present within the cytoplasm of both dormant and germinating ascospores. Exposure of sections to the electron beam caused these structures to become empty indicating that volatilization of the vacuolar contents occurred. It is significant that such vacuoles were never found to be present within the limits of the nuclear membrane. The lipoidal nature of these vacuoles could be demonstrated by staining the ascospores with Sudan black B without any prior treatment (figure 3).

Although there are no indications of the presence of mitochondria in the resting ascospore, small granules of various sizes are found scattered in the periphery of the ascospore. Although these structures are observed in the original electron micrographs, their presence is obscured in subsequent reproductions. The surprisingly early appearance of recognizable mitochondria (figures 4 and 6) in the peripheral region of the germinating ascospore suggests the transformation of these granules into mitochondria early in the germination process.

Various inclusions were also observed in the ascus cytoplasm (figure 1) each being characterized by a definite limiting membrane. These were also stainable with Sudan black B.

Ascus wall. The nature of the ascus wall is essentially the same as reported by other investigators (Northcote and Horne, 1952; Bartholomew and Levin, 1955). No remnants or vestiges of the cytoplasmic membrane were observed to be associated with the ascus wall.

Germination process. When placed in the germination medium, the ascospores swelled resulting in breakage of the outer spore coat. Usually breakage occurred at more than one point, indicating a lack of ductility. In contrast to this, the inner spore coat was resistant to disruption. Simultaneously with the swelling process, even prior to breakage of the outer spore coat, typical mitochondria enclosed in double membranes, with observable *cristae mitochondriales*, were formed. Although the shapes of the mitochondria were variable, neotetrazolium salts

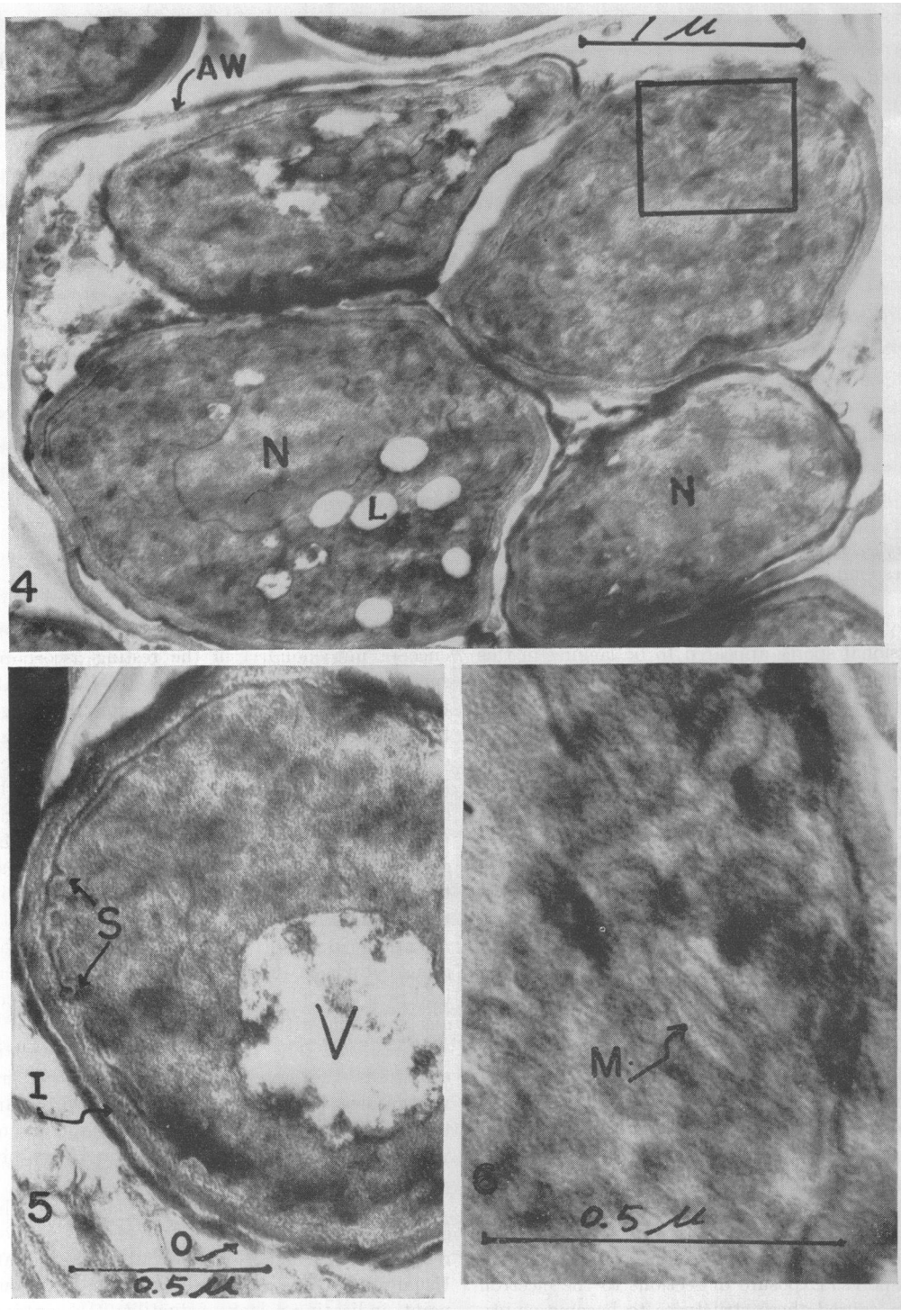


Figure 4. An early stage of ascospore germination. The preparation was treated with lanthanum nitrate. Note the considerable increase in electron density of the inner ascospore coat and the cytoplasmic membrane.

Figure 5. A lanthanum nitrate treated section of a germinating ascospore. Note the invaginations of the cytoplasmic membrane and the appearance of a large vacuole in the cytoplasm.

Figure 6. An enlargement of a portion of figure 4 demonstrating the presence of a mitochondrion with typical cristae.

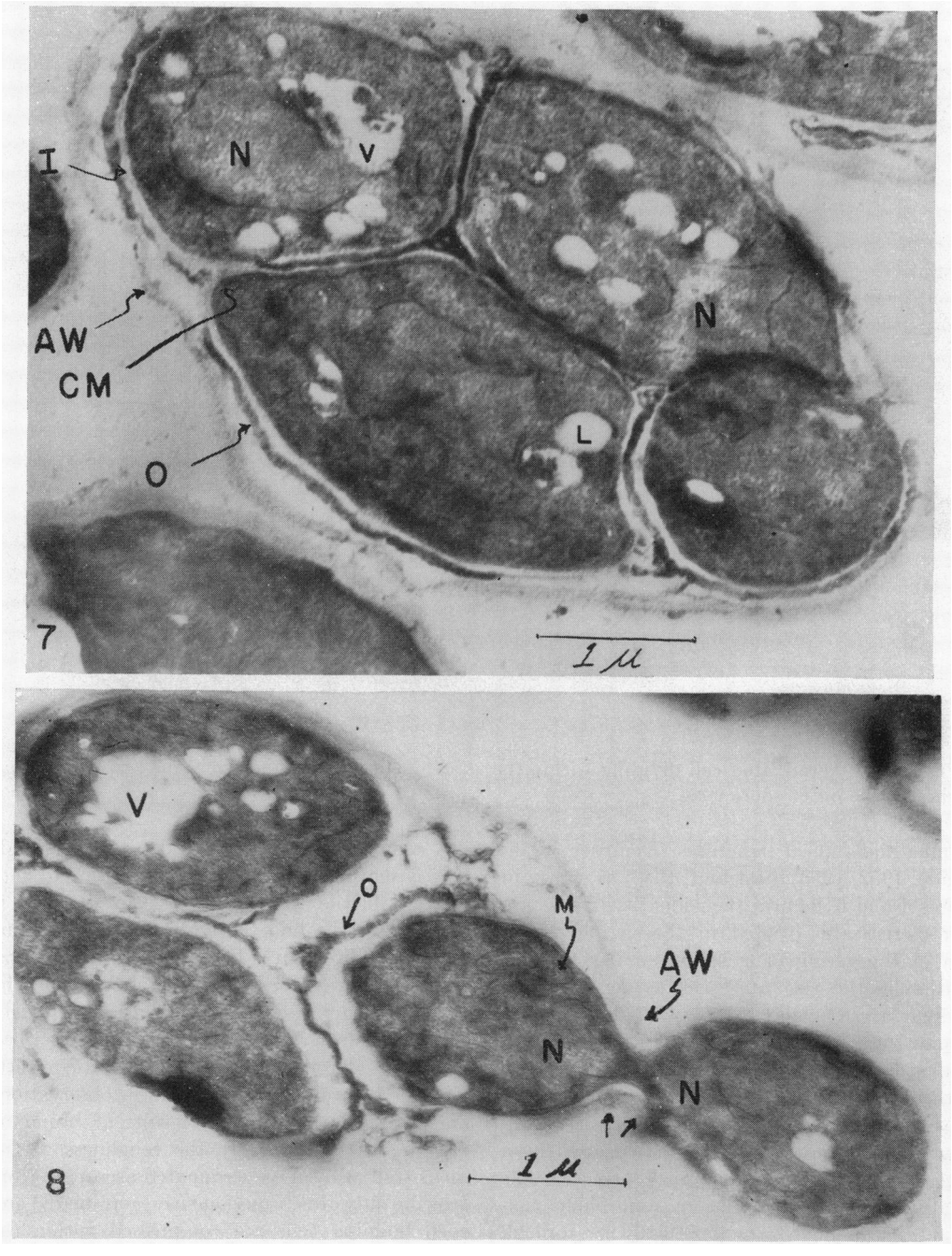


Figure 7. Germinating ascospores showing typical swelling within the ascus. Compare the size and shape with the dormant ascospores shown in figures 1 and 2.

Figure 8. A typical budding ascospore. The bud is carrying part of the ascus wall outwards (indicated by the double arrows).

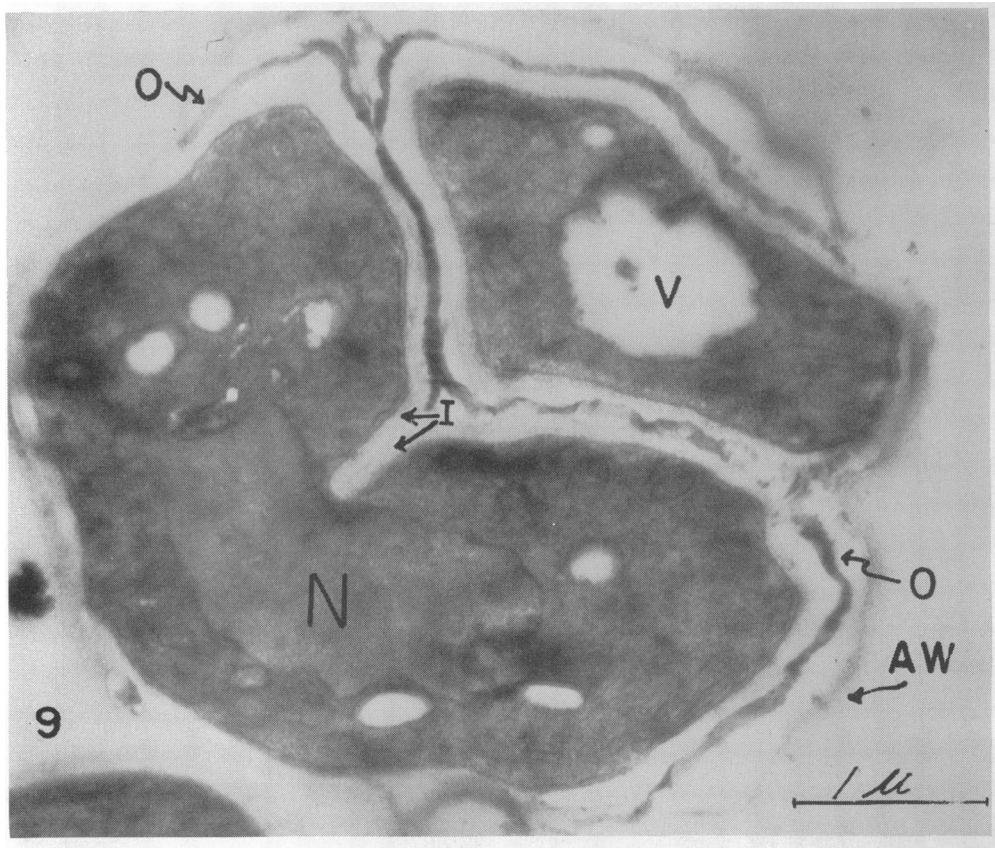


Figure 9. A germinated cell dividing within the ascus. Note the prominent nucleus and the circumference of the outer spore coat.

staining and typical structures (Dempsey, 1956; Palade, 1952, 1953; Sjöstrand, 1953) as shown in figures 4 and 6 confirm their identity. These are characteristically located in the peripheral region of the germinating spore, similar to their position in the vegetative cell as demonstrated by Agar and Douglas (1957). Usually one large vacuole (figures 5, 8, and 9) in addition to several smaller ones was formed next to the nucleus early in the germination process. This occurred even prior to shedding of the outer spore coat. Although each type of vacuole was enclosed by a single limiting membrane, the large vacuole usually contained granular or amorphous material. This vacuole characteristically was formed, and remained, in close proximity to the nucleus.

The inner spore coat of low electron density gives rise to the new vegetative cell wall which is also of low electron density. The density of the inner spore coat of the germinating ascospore is remarkably increased by treatment with lanthanum nitrate (figures 4 and 5).

A completely germinated spore initiating budding is shown in figure 8. It is interesting to note that the ductile nature of the ascus wall makes it possible for the bud to carry part of the ascus wall in the direction of bud formation. No alteration in internal or external structure of the nucleus occurred during germination. Changes occurring during bud formation will be described elsewhere. Chromosomal or characteristic mitotic structures were not observed. These observations are in agreement with the results of Mundkur (1954). Not infrequently the toughness of the ascus wall prevents a germinated ascospore from growing outwards. Consequently, germinated and even budding cells are compressed within the ascus wall resulting in deformation. Figure 9 demonstrates this effect. It is quite clear from the circumference of the shed outer ascospore coat that this is not a conjugation figure. Although the conjugation of the two germinated ascospores remains a possibility no convincing electron micrographs were obtained.

DISCUSSION

Although many details of the basic structure of the bacterial endospore have been obtained from recent studies using ultrathin sectioning techniques and electron microscopy, little is known of the internal structure and organization of the yeast ascospore. Since yeasts are classified higher than bacteria on the evolutionary scale, comparison of endospore and ascospore structure presents some intriguing possibilities. These spores have certain characteristics in common which differ only in degree, such as heat resistance and resistance to staining with basic dyes. Comparison of spore structure may offer an opportunity for correlation of structure and physiology.

Previous attempts to observe the fine structure of *S. cerevisiae* ascospores were not successful due primarily to technical difficulties. Bartholomew and Mittwer (1953, 1954) presented electron micrographs of *S. cerevisiae* ascospores subjected to ultraviolet photolysis for 72 hr. These authors attempted to demonstrate the presence of a single nucleus and other related structures within the ascospore. As pointed out by Mundkur (1954), ultraviolet photolysis is a rather drastic treatment yielding ample opportunity for the production of artifacts. The dense bodies observed in their micrographs may well be due to shrinkage and disruption of the cytoplasm. Agar and Douglas (1957) using ultrathin sectioning and electron microscopy concluded that "Asci are quite susceptible to disruption during polymerization. . . . Three membranes surrounding the spore can be seen but the spore itself appears dense and lacking in internal structure." McClary *et al.* (1957) demonstrated the presence of a single nucleus in individual ascospores by means of an improved Carnoy-perchloric acid-Giemsa staining method; however, no unambiguous nuclear structure has been demonstrated in electron micrographs of *S. cerevisiae* ascospores.

In reference to the mode of germination, Skinner *et al.* (1951) pointed out that "some yeast ascospores had a double membrane and on germination the outer membrane was ruptured and cast off, the inner membrane serving as the cell wall of the new vegetative cell." Here again, the mode of germination has not been studied or confirmed by means of ultrathin sectioning and electron microscopy. To our knowledge no critical work has been done on the development of mitochondria and other structures during germination of the ascospore.

The advantage of obtaining serial sections for the demonstration of a specific structure is evident in figures 1 and 2. Structures which might appear to be lacking, or completely absent from an ascospore simply due to the plane of sectioning can be easily observed if more than one section of an individual cell is obtained. However, extreme caution must be used in attempting to elucidate the three dimensional figure of a structure from serial sections as demonstrated by Williams and Kallman (1955).

A series of comparative studies was undertaken in an attempt to identify the nucleus, since the identity of this structure is a subject of much controversy. The structure and behavior of various organelles during germination were followed using cytochemical techniques as well as electron microscopy. These studies supported the contention that *N* in figures 1 and 2 is the nucleus of the ascospore. It is interesting to note that the yeast nucleus, like the bacterial nuclear apparatus (Chapman and Hillier, 1953) appears to be much less electron dense than does the cytoplasm, whereas nuclei of animal tissue cells appear much more electron dense than the cytoplasm. The distribution of electron dense material within these various cells probably reflects differences in cytoplasmic and nuclear composition and may be significant. However, loss of diffusible material during specimen preparation remains as a possibility. Although the ascospore nucleus, in common with nuclei of higher organisms, is surrounded by a characteristic double membrane, no typical chromosomal figures were observed in any of the electron micrographs. Such structures have been demonstrated in the nuclei of animal cells (Borysko, 1953; Fawcett, 1956, De-Robertis, 1956) using the ultrathin sectioning technique. The presence of a variable number of inclusions within the cell (which stain with Sudan black B) may cause confusion when attempting to follow the location and behavior of the nucleus by light microscopy (figure 3). These inclusions are structurally distinct from other structures within the cell, especially when observed in electron micrographs. It is quite significant that the presence of these structures (*L* in figures 1, 2, 4, and 7) is exclusively limited to the cytoplasm. Unpublished electron micrographs of *Nadsonia fulvescens* ascospores revealed the presence of a large lipoidal vacuole which

also stained with Sudan black B and was structurally identical, except for size, to the vacuoles observed in *S. cerevisiae* ascospores.

Although there are no definite structures which can be identified as mitochondria in the dormant spore, the rapid appearance of typical mitochondria (*M* in figure 6) in the cytoplasm upon transfer of the spore to the germination medium suggests that there is a precursor of the mitochondrion, or preformed subunits within the dormant ascospore. The typical mitochondria appear even before rupture of the outer spore coat, which tends to favor the subunit theory. In this connection Dempsey (1956) pointed out the inadequacy of the "fission" theory of mitochondrial multiplication and development in higher animal cells. He also contended that the theory of fabrication of new mitochondria from intracellular membranes in higher animal tissues is not adequately substantiated. There are indications, but no direct evidence that the structures occasionally seen in the ascospore cytoplasm are structural precursors of mitochondria.

The structures (*G* in figure 1) which occur within the ascus wall, although not enclosed within the ascospore, stain deeply with Sudan black B, each being surrounded by a thick limiting membrane. These granules in common with vegetative cell mitochondria, are lipoidal in nature (Hartman and Liu, 1954; Sarachek and Townsend, 1953; Lindegren, 1949). Therefore it seems logical to conclude that these structures are only remnants of the mother cell mitochondria since *cristae mitochondriales* appear to be absent. These structures may, however, be part of the endoplasmic reticulum.

It is also interesting to note the apparent invagination of the cytoplasmic membrane in the dormant spore. The number of invaginations appears to increase as the spore becomes metabolically active, which is readily understandable when the physiological function of the cytoplasmic membrane is taken into consideration. The cytoplasmic membrane appears as a sinuate structure in the vegetative cell which is in agreement with results obtained by Agar and Douglas (1957). This structure is most clearly demonstrated in preparations treated with lanthanum nitrate. The reason for the affinity of this structure for lanthanum nitrate, and the difficulty in demonstration of its presence in preparations not treated with this reagent is not known at present.

The inner spore coat of the ascospores, and the cortex of spores of *Bacillus megaterium* (Mayall and Robinow, 1957) have the same low electron density and affinity for lanthanum nitrate. Consequently, the endospore cortex and the inner

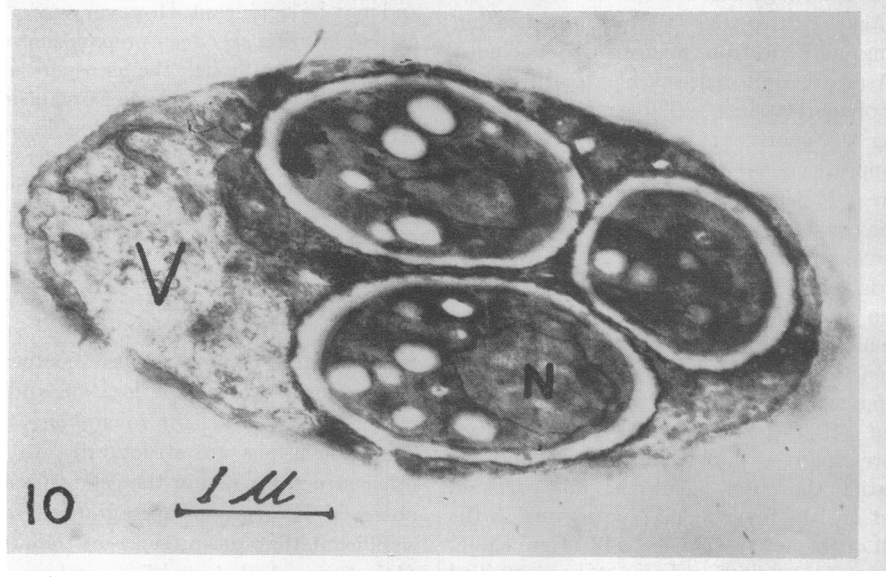


Figure 10. An ascus which is presumed to have completed sporulation. Note that the vacuole, which is readily observed in vegetative cells, is completely excluded from the ascospores. The presence of three spores instead of four is probably due to the plane of sectioning.

coat of the ascospore have a similar appearance when exposed to the electron beam or to lanthanum nitrate. Since there are no published data on the composition of the ascospore wall, nor the cortex of the *B. megaterium* endospore, it is difficult to draw any concrete conclusions. However, these common characteristics may be reflected in common functions, such as resistance to basic dyes. The appearance of a similar zone in *Clostridium sporogenes* was correlated with increased resistance to basic dyes (Hashimoto and Naylor, 1958*b*). Future studies in this area might serve to identify the common constituent(s) within these structures responsible for lanthanum nitrate affinity, and possibly its physiological role in the dormant spore.

The present investigation clearly establishes the formation of the cell wall directly from the inner spore coat in *S. cerevisiae* which supports the contention of Skinner *et al.* (1951) that in some yeast the inner ascospore wall serves as the cell wall of the new vegetative cell. Recent studies by ultrathin sectioning reveal that a similar process occurs in *Bacillus cereus* (Chapman and Zworykin, 1957) and *B. megaterium* (Mayall and Robinow, 1957). Although other investigators (Preisz, 1919; Knaysi *et al.*, 1947) reported that the cell walls of bacteria are formed anew during germination, it now seems clear that the vegetative cell wall of germinating bacterial and yeast spores is formed from a preexisting inner spore coat.

Prior to rupture of the outer ascospore coat, the formation of a large vacuole (*V* in figures 5, 8, 9, and 10) within a definite limiting membrane becomes apparent. This vacuole is formed in close proximity to the nucleus, does not stain with Sudan black B, appears to have a network of fine fibers in aged cells, and is easily distinguishable from other inclusions. This structure persists throughout the vegetative cell phase. The absence of this vacuole from the dormant ascospore, complete separation from the nucleus by definite limiting membranes in sections of vegetative cells (Agar and Douglas, 1957) and the fate of this vacuole during sporulation (complete exclusion from the ascospore) (figure 10), supports the view that this structure is not an integral part of the nucleus.

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SUMMARY

The fine structure of dormant and germinating ascospores of *Saccharomyces cerevisiae* was studied by means of ultrathin sectioning and electron microscopy. Structure and behavior of the nucleus, mitochondria, and various inclusions were followed during germination by cytochemical techniques as well as electron microscopy. The ascospore is surrounded by two coats, the inner one giving rise to the cell wall of the vegetative cell during germination. Origin of mitochondria, and basic similarities in the structure of bacterial endospores and yeast ascospores were discussed. The nature of the large vacuole regularly found in vegetative cells was also discussed. Evidence was presented indicating that this structure is not an integral part of the nucleus.

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