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Ultrastructural Changes of the Fission Yeast *(Schizosaccharomyces pombe)* during Ascospore Formation*

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Summary. The fission yeast, *Schizosaccharomyces pombe*, a homothallic haploid strain NCYC 132, was induced to flocculate and conjugate to facilitate the study of the ascosporogenesis. It is found that two nuclei are formed during meiosis I and these nuclei divide again during meiosis II. The forespore membrane emerges at the beginning of meiosis II, elongates without fragmentation to enclose the nucleus and other cytoplasmic organelles, including mitochondria and ER. Meiosis occurs without fragmentation of the nuclear membrane. Immediately after the enclosure of the nucleus, the forespore membrane is resolved into two separate membranes. The inner one appears to become the spore plasmalemma and the outer one, if it persists, becomes a limiting membrane of the spore wall. In some cases, the outer membrane is seen to be ruptured. Spore wall materials deposite in the space between the inner and outer membranes.

Ultrastructural changes during ascoporogenesis have been studied in ascomycetes using both conventional chemical fixation (Bandoni *et al.*, 1967; Black and Gorman, 1971; Carrol, 1967, 1969; Conti and Naylor, 1959, 1960a, b; Hashimoto *et al.*, 1960; Lynn and McGee, 1970) and freeze-etching preparations for electron microscopy (Black and Gorman, 1971; Guth *et al.*, 1972). Ascosporogenesis comprises a series of events which include karyogamy, meiosis, spore membrane formation, and spore maturation. Although the basic outline of events has been established (see Carrol, 1969; Fowell, 1969), the details of

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ascosporogenesis tend to vary from one species to another among the ascomycetes (Bandoni *et al.*, 1967; Black and Gorman, 1971; Carrol, 1967, 1969).

The fission yeast, *Schizosaccharomyces octosporus* has been the subject of investigations on mitosis and ultrastructural changes both preceeding (Conti and Naylor, 1959, 1960a) and during the ascospore formation (Conti and Naylor, 1960b). McCully and Robinow (1971) have recently studied mitosis in another fission yeast, S. *pombe*, but no studies have been reported on the ascosporogenesis in *S. pombe*.

Materials and Methods

Culturing Techniques. Schizosaccharomyces pombe NCYC 132, a homothallic haploid strain, was used for the study. Cloned cells of the strain were cultured as described previously (Calleja and Johnson, 1971). Briefly, stationary phase cells were reinoculated into 10 ml Malt Extract Broth (2% Oxoid) and allowed to grow in a sealed 1 oz. bottle until the stationary phase had been reattained. This culture was then poured into an open 125 ml conical flask and shaken at 150 rpm to induce flocculation. Flocculation occurs only after the logarithmic phase of growth, and is prerequisite to conjugation. The first signs of conjugation appeared about $2^1/_2$ to 3 h after induction of flocculation, and sporulation became evident about 7 h later.

Electron Microscopy. At different times after induction of flocculation, flocculated cells were separated from the noninduced free cells by differential sedimentation. The flocs were then briefly washed in distilled water and fixed in freshly prepared aqueous 2% $KMnO_4$ at room temperature for 1 h. The fixed cells were dehydrated in acetone and embedded in a mixture of epon and araldite (Mollenhauer, 1964). Thin sections were stained in lead citrate (Reynolds, 1963) and examined with a Philips 200 electron microscope.

Fig. 1. Electron micrograph of a fission yeast zygote at the beginning of meiosis II. In the cytoplasm are seen two haploid nuclei (*N*), which are the product of meiosis I, mitochondria (*M*) in various shapes, ER parallel to the plasmalemma, short fragments of ER, and the forespore membrane (*FM*) (arrow). ×14400

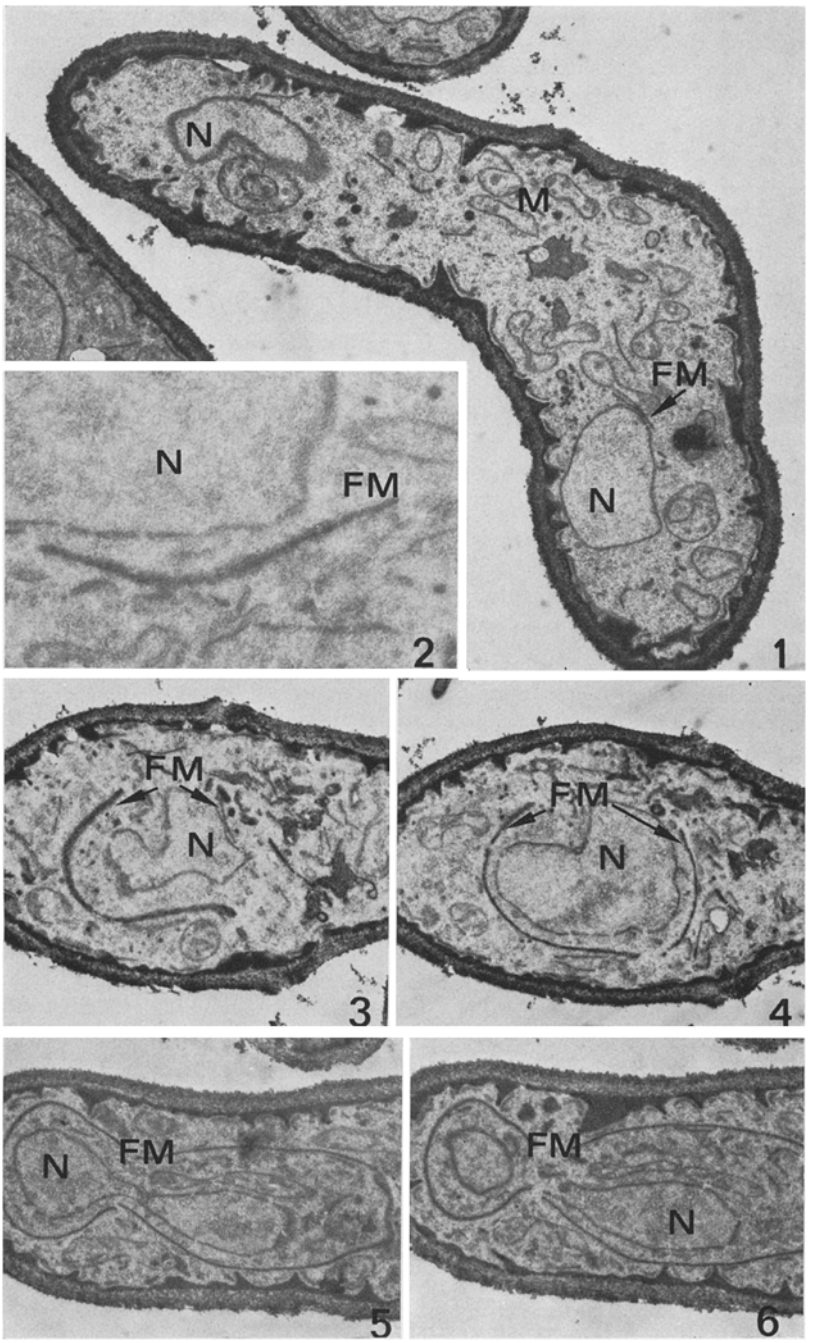
Fig. 2. Electron micrograph showing the forespore membrane (*FM*) proximal to the nucleus (*N*). In comparing the *FM* with the nuclear membrane and ER, the *FM* can be easily identified by its location, electron-density, thickness, and length. ×35700

Fig. 3. The *FM* has encircled half of the nuclear circumference. Note the shape of the nucleus (*N*) and the second *FM* in its incipient stage of development (arrow). Also note an increased distance between the nucleus (*N*) and the *FM*. The *FM* appears to be sectioned slightly obliquely. ×13100

Fig. 4. Two *FM*s have almost completely encircled the nucleus (*N*). Note a constriction of the nucleus at the central zone. ×13100

Fig. 5. Electron micrograph showing the process of meiosis II. Two *FM*s have encircled the nucleus (*N*) which has elongated and constricted at the center. Note the presence of the nuclear membrane. ×15100

Fig. 6. A serial section of Fig. 5. The comparison enables visualization of the nucleus as being dumbbell-shaped. ×15100



Figs. 1—6

The micrographs were assembled in order that the sequence of events might be reconstructed. Studies on flocculation will be reported elsewhere. We deemphasize many of our observations which parallel those of others (Black and Gorman, 1971; Carrol, 1967, 1969; McCully and Robinow, 1971) on other ascomycetes. Hence our account begins at the end of Meiosis I.

Results

Following karyogamy and meiosis I, one finds that the incipient ascus contains two the separated nuclei (Fig. 1). The forespore membrane ["prospore wall" in Moens' terminology (1971)], which ultimately will delimit the spores, emerges after meiosis I and before constriction of the haploid nuclei. At this stage of development the forespore membrane (FM) is difficult to distinguish from endoplasmic reticulum (ER) or other cytoplasmic membranes (Fig. 1). However, when it can be identified as the FM, it has already attained the following characters: 1. It is proximal to the nucleus. 2. It is more densely stained than other cytoplasmic membranes, including ER (Figs. 2—9). 3. It is thicker and longer than other cytoplasmic membranes (Figs. 2—9).

Later the distance between the FM and the nucleus increases slightly and the FM begins to elongate (Figs. 3 and 4). While the extending FM begins to curve around, as though to encircle the nucleus, a second FM is initiated at the periphery of the nucleus at the opposite pole (Fig. 3). Although the initiation and development of one FM always precedes the other, it becomes difficult at the later stages of development to distinguish which was the first. Eventually each FM approximates the shell of a hemisphere encircling the nucleus.

By this time the nucleus is seen to be "pinched in" (Fig. 3). The nucleus continues to elongate while constricting at the midregion.

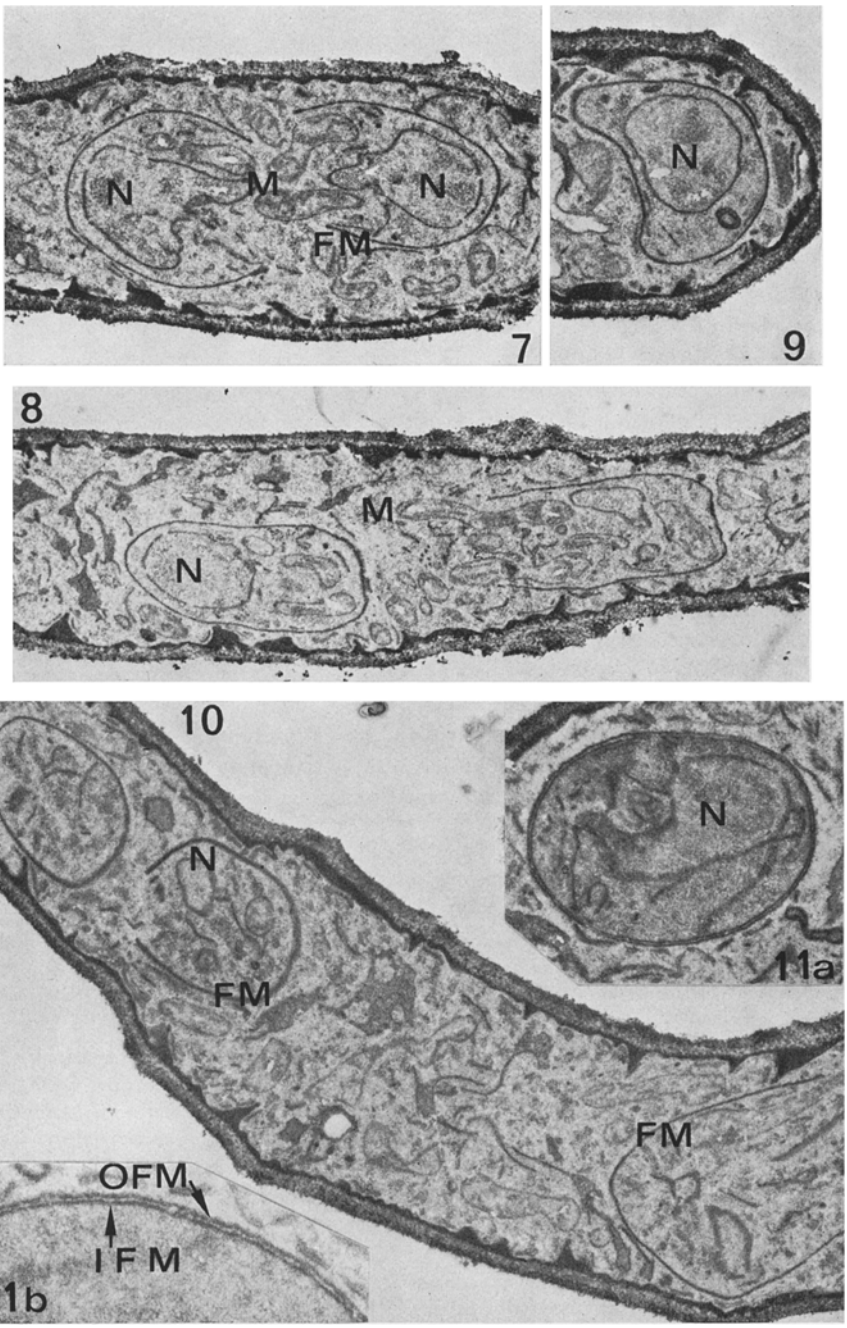
Fig. 7. Electron micrograph of a cell in which meiosis II has just been completed. Two daughter nuclei (*N*) are almost entirely encircled by their *FM*s. Note mitochondria (*M*) engulfed by the *FM*. ×15100

Fig. 8. One of two daughter nuclei (*N*) is now completely enclosed by the *FM*, while the other seems to be in the process of complete enclosure. Mitochondria (*M*) appear to flow into the inside of the *FM*. ×15100

Fig. 9. Electron micrograph of an "ascospore" without the wall. The *FM* is not resolved into two membranes. Nucleus (*N*). ×15800

Fig. 10. Electron micrograph showing nuclei (*N*) in the process of encirclement by their *FM*s and emphasizing the asynchrony of the process. This photograph also demonstrates that meiosis I produces two nuclei which, in turn, divide again during meiosis II. The *FM* surrounding the two ascospores at left appears to be sectioned slightly obliquely. ×15100

Fig. 11. a The FM is now resolved as two membranes. ×19900. b High magnification of the area enclosed in a rectangle in an outer forespore membrane (*OFM*). Inner forespore membrane (*IFM*). ×35700



Figs. 7—11

Serial sections (Figs. 5 and 6) indicate that the nucleus is dumbbell-shaped. The FM extends concomitantly (Figs. 5 and 6).

Meiosis II in *S. pombe* proceeds without fragmentation or loss of the nuclear membrane, which merely seems to elongate before constriction of the nucleus. As a consequence of nuclear division and the continued extension of the FM, each new nucleus is substantially encircled by FM (Fig. 7). The FM finally encloses the nucleus and along with it, mitochondria, ER, and other cytoplasmic organelles (Figs. 7—9). Mitochondria even appear to "flow" into that cytoplasm delimited by the FM (Fig. 8).

The process of nuclear enclosure is not synchronous. When the one first nucleus is completely enclosed by the FM, other nuclei of that ascus are still at various stages of enclosure. This asynchrony seems to be the norm rather than the exception, for we have never observed any two nuclei of one ascus in the same stage of delimitation.

The completely enclosed nucleus and cytoplasm plus FM constitute an "ascospore" without spore wall. However, development of the spore wall soon follows. The deposition of spore wall materials does not appear to proceed at a uniform rate among the spores of the same ascus (Fig. 15), and this is further evidence for asynchronous spore development.

The cytoplasm of the spores gradually becomes even more densely stainable, as the spores attain maturity, until finally the cytoplasm stains so densely that no cellular organelles are discernable (Fig. 16). However, mitochondria and other cytoplasmic organelles are obviously present within the spore up to that point.

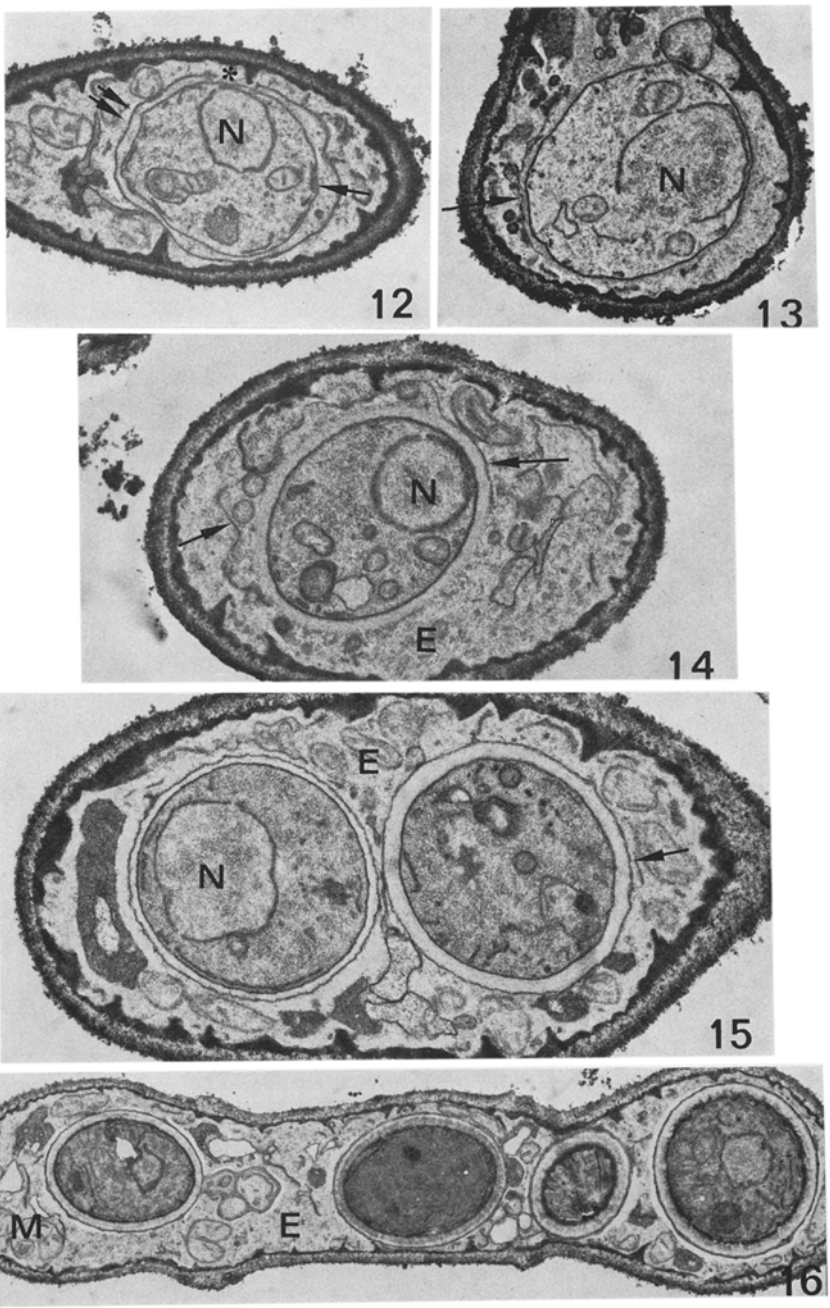
Fig. 12. Electron micrograph of a later stage in ascospore formation. Spore wall materials have deposited in the intercisternal space. The outer membrane (double arrow ⇉) is now further separated from the inner one (single arrow →). An asterisk (*) indicates a point where the outer membrane is discontinuous. The cytoplasm of the spore is seen to contain mitochondria and ER. ×22800

Fig. 13. All but a few fragments of the outer membrane have disappeared. Arrows indicate fragments of remaining outer membrane. ×13700

Fig. 14. The outer membrane of the *FM* seems to be further pushed away from the inner one by an increased deposition of the wall materials. The arrows indicate the outer membrane. Epiplasm (*E*). Nucleus (*N*). ×20800

Fig. 15. Two ascospores are in process of maturation. [Both the outer and inner membranes of the *FM* are seen as single membranes (arrow).] Note the difference in the thickness of the spore walls. Epiplasm (*E*). Nucleus (*N*). ×22800

Fig. 16. Electron micrograph of an ascus containing 4 ascospores, each of which appears to show a different degree of maturation. Note persistant mitochondria (*M*) in the epiplasm (*E*) and in some spores. ×11500



Figs. 12—16

Discussion

Schizosaccharomyces pombe NCYC 132, a homothallic haploid strain, produces usually four ascospores per ascus after conjugation. However, most of our thin section have shown only two and consequently our observations are largely confined to the formation and development of two ascospores. Two reasons account for this: 1. *S. pombe* zygotes are not straight, but bent at the point of fusion. 2. According to Calleja and Johnson (1971), about 50% of the ascus population formed under the same experimental conditions as those described above contained one to three ascospores in an ascus.

As reported in other ascomycetes studied (Guth *et al.*, 1972; Moens, 1971), the FM emerges at the beginning of meiosis II in *S. pombe* (Figs. 1—4). As to the origin of the FM, the literature is divided and inconclusive. For example, Carrol (1967, 1969) has observed that blebs were derived from the nuclear membrane and fused to form the FM in *Saccobolus*. On the other hand, some have suggested that the ER and/or other cytoplasmic membranes, including the ascus plasmalemma, are the origin of the FM (Black and Gorman, 1971; Conti and Naylor, 1960b; Lynn and McGee, 1970; Syrop and Beckett, 1972).

In *S. pombe*, however, the FM appears to be originated from the nuclear membrane. Whenever the FM has been identified at the beginning of meiosis II, it has invariably been located very close to the nuclear membrane. Unlike the ER and other cytoplasmic membranes the FM continues to elongate without fragmentation during meiosis II, encircles the nucleus, and shows a close co-ordination with meiosis II and subsequent events. It is clearly an organelle with specialized functions.

Although the FM can be easily identified by its electrondensity, length, and thickness (Figs. 2—9), it is difficult to differentiate the FM from other cytoplasmic membranes at its incipient stages of development (Fig. 1). For this reason, the origin of the FM in *S. pombe* is yet to be discovered and, to this end, both the conventional chemical fixation and freeze-etching electron microscopy seems to be inadequate.

No serious attempt was made to determine if the FM is made of a single or double membrane during early stages of development, but, because of its thickness, it would seem to be double membranes. Later it becomes evident that the FM is composed of double membranes (Fig. 11).

Once segregation of the two membranes has occurred, each membrane follows a different path of development. As reported in other ascomycetes (Bandoni *et al.*, 1967; Black and Gorman, 1971; Hashimoto *et al.*, 1960), the inner membrane seems to become the spore plasmalemma in *S. pombe* (Figs. 11—13 and 15), while the outer one is separated from the inner

one, as spore wall materials deposite in the intercisternal space, and becomes, if it persists, the outer limiting layer of the spore wall (or the investing membrane). However, we believe that it is ordinally spore wall materials and not the membrane that constitutes the outermost layer of the mature spore wall.

In the present study, an increase in the distance between the inner and outer membrane is taken as the growth of the spore wall, and the site of the deposition is assumed to be in the intercisternal space. Using a silver methenamine staining, Black and Gorman (1971) have demonstrated that the intercisternal space is the site of polysaccharide deposition.

Cellular organelles in the epiplasm (cytoplasm of the ascus) (Figs. 12—16), especially the mitochondria, appear to persist to a quite advanced stage of spore maturation. Judging from both the persistence of mitochondria in the epiplasm and their ultrastructure, the epiplasm seems to remain metabolically active for a long time after the delimitation of spores. As suggested by others (Bandoni *et al.*, 1967; Lynn and McGee, 1970), the epiplasm may indeed participate in the process of the synthesis of the wall materials.

As shown above (Figs. 3—9), meiosis II in *S. pombe* proceeds without dissolution of the nuclear membrane. Unlike in *S. cerevisiae* (Guth *et al.*, 1972; Moens, 1971) where the nucleus remains incompletely divided during meiosis I and II, meiosis I and II in *S. pombe* are clearly separated by time: two haploid daughter nuclei are produced at the end of meiosis I followed by another division of these nuclei during meiosis II (Figs. 1—9).

It is indeed surprising to find that ascosporogenesis in *S. pombe* is an asynchronous process in that the development of each ascospore in the same ascus proceeds at a different rate. The asynchrony is true for the initiation and subsequent development of the FM (Figs. 3 and 4), enclosure of the nucleus by the FM, deposition of the spore wall materials (Fig. 15), and finally, for the ultimate maturation of the spores.

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