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Electron Microscopy of Cell Fusion in Conjugating *Hansenula wingei*

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ABSTRACT

CONTI, S. F. (Dartmouth Medical School, Hanover, N.H.), AND T. D. BROCK. Electron microscopy of cell fusion in conjugating *Hansenula wingei*. J. Bacteriol. **90**:524–533. 1965.—The heterothallic yeast *Hansenula wingei* is a favorable organism for the study of the process of cell fusion, since strong agglutination of cells of the two mating types ensures a high percentage of cell fusions. The initial agglutination reaction results in cell-wall deformation, so that the walls in the region of contact are tightly appressed over an extensive area. The fusion process is initiated when the walls of two cells elongate, and this elongation seems to be restricted to the region where the cells touch. Occasionally, one cell is seen to push in the wall of the other, but in many cases both cells elongate equally, as would be expected in an isogamous organism. The precise disposition of the elongating wall probably reflects the manner in which the cells initially become associated in the agglutinated cell clump. Soon after wall elongation begins, cell-wall fusion occurs along the margin of contact. Only after fusion is complete is the wall separating the two cells dissolved away. If wall dissolution begins at one edge of the conjugation tube, a flap is formed in which can be seen the remnants of the fused walls. Alternatively, dissolution can begin at the center of the conjugation tube, proceeding towards the outside. Conjugating cells are uninucleate, and the nuclei are large and frequently lobed or elongated. After the conjugation tube is formed, the nuclei migrate towards the center, and fusion occurs only over a small region where the nuclear membranes come in contact. After nuclear fusion, the first diploid bud forms from the conjugation tube and at right angles to the tube axis. The diploid nucleus then migrates into this bud. Frequently, in the later stages of conjugation, a large vacuole develops in each of the original cells. All of the above events will occur in a medium devoid of a nitrogen source and in which vegetative budding will not occur.

Cell fusion is a phenomenon which occurs frequently in organisms with cell walls, both in vegetative and in sexual processes. In plants, cell fusion occurs in the conversion of rows of xylem cells into xylem vessels, in the formation of sieve tubes in phloem, and in the formation of articulated laticifers (Frey-Wyssling, 1959); hyphal fusions occur among vegetative cells in many fungi (Buller, 1933). The clamp connection, a characteristic structure which forms at each cell division in dicaryotic basidiomycetous fungi, also arises by means of a fusion process (Buller, 1933). In addition, cell or hyphal fusion is a routine event of the sexual cycle in fungi and algae (Sassen, 1962), and fusion may also have its analogy in pollination processes in higher plants. The formation of haustoria by plant pathogenic fungi may also occur by a process analogous to cell fusion.

Despite its widespread importance, cell fusion in most systems is difficult to study either biochemically or ultrastructurally, since usually only a minute proportion of the protoplasmic mass or cell population is undergoing fusion at any given time. In the sexually agglutinative yeast *Hansenula wingei*, the fusion process can be studied under quite favorable conditions. All of the vegetative cells of this yeast are able to function as gametes, and, when suspensions of the two mating types are mixed, up to 80% of the cells fuse in pairs. This fusion will occur in a reasonably synchronous manner, and in a medium lacking nitrogen source and essential vitamins, in which vegetative growth does not occur (Brock, 1961). It is thus possible to readily prepare material for electron microscopy in which all stages of the

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conjugation process can be observed. Although it is unlikely that the events observed in this yeast are identical in detail to the fusion processes occurring in other plants, it is reasonable that the general outlines of the process will be similar, so that the present work may provide a model with which other systems can be compared.

MATERIALS AND METHODS

Cultures. H. wingei NRRL Y-2340, strains 5 and 21, the agglutinative mating types, had been obtained in 1957 from L. J. Wickerham, and they have been kept in vegetative culture since then with no diminution in their sexual powers.

Growth and conjugation. The cells were grown into the stationary phase (2×10^8 to 4×10^8 per milliliter) on a rotary shaker at 30 C in a liquid medium of the following composition: glucose, 30 g; yeast extract (Difco), 7 g; KH_2PO_4, 5 g; water, 1,000 ml; *p*H 5.5. The cells were then washed twice in water and suspended in water, and the cell densities of the two strains were adjusted to be equal. The two suspensions were then mixed, $MgSO_4$ was added to 0.1% (w/v), the mixture was centrifuged in a clinical centrifuge, and the now agglutinated cell pack was suspended to give a final cell concentration of about 3×10^8 per milliliter in a medium of the following composition: KH_2PO_4, 0.01 M; $MgSO_4 \cdot 7H_2O$, 0.1%; glucose, 0.5%; *p*H 5.5. The agglutinated suspension was then placed on a rotary shaker at 30 C, and samples were removed at various intervals.

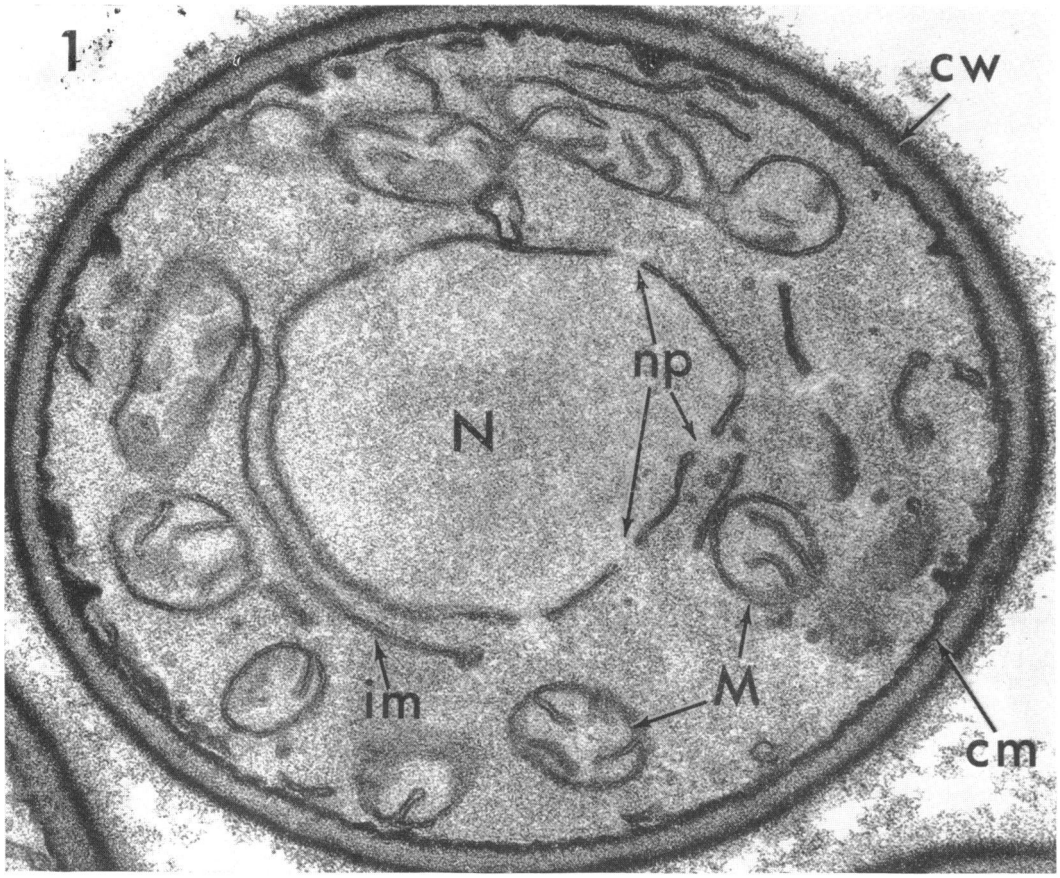
Fixation for electron microscopy. Samples (1 ml) were centrifuged, and washed twice in water. The pellet was suspended in 1 ml of 1.5% potassium permanganate, and was kept at room temperature for 20 min; the fixed cells were washed once in water, suspended in 1 ml of 2% osmic acid for 60 min at room temperature, and washed twice in water. The pellets were then suspended in one or two drops of warm 2% water-agar, and the still-warm suspension was spread in a layer 1 mm thick on a microscope slide. After hardening, the agar was cut into 1-mm cubes, which were treated with 50% aqueous ethyl alcohol (v/v) for 30 min and then transferred to 70% ethyl alcohol. The specimens were left in 70% alcohol for 2 to 5 days, and were then completely dehydrated and embedded in Epon 812 essentially as described by Luft (1961). Sections were cut with a diamond knife on an LKB ultratome and examined in a Phillips EM-200 electron microscope.

RESULTS

Cell-wall deformation during sexual contact. As noted in the initial work on *H. wingei* (Brock, 1958), the attraction between cells of opposite mating type is so strong that the walls actually become deformed. Figure 1 shows a thin section of a normal unagglutinated cell of *H. wingei*, and Fig. 2a shows a thin section through a portion of an agglutinated mass. In the latter, the walls are very tightly appressed. In this clump it appears as if two cells of the same mating type are in intimate contact, since the three central cells in the picture are all in contact. It is likely, however, that this phenomenon is an artifact resulting from the strength of the agglutination reaction, two cells of like mating type being forced passively together, since it has been clearly shown (Brock, 1958) that only cells of opposite mating type are able to interact specifically with each other. When only a few cells of opposite mating type are brought together, cell-wall deformation still occurs, although less drastically, as shown in Fig. 2b. If an agglutinated mass of cells is deagglutinated under conditions which preserve all viability and conjugability, most of the cells return to their normal ovoid shape, as seen in the light microscope (Brock, *unpublished data*). Thus, it is likely that the *H. wingei* cell wall possesses considerable pliability in the face of the stresses attendant the agglutination reaction.

Formation of the initial conjugation tube. In the light microscopical studies (Brock, 1961), it was shown that the wall of each cell of a conjugating pair elongated in the region where the two cells touched. Similar observations have now been made in the electron microscope, although the exact disposition of the elongating portions probably depends at least to some extent on how the cells were initially united in the agglutinated clump. In Fig. 3, the cell-wall elongation of both cells has taken place in a plane other than the long axis, so that the cells have become distinctly misshapen; on the larger cell, the protrusion which is not in contact with the other cell is not the beginning of another conjugation tube, but a bud scar. Figure 4 shows an example in which the two cells have come in contact so that they touch at their poles, and both cells have elongated equally.

Cells such as those of Fig. 5a and 5b have been observed, in which the elongating protuberance of one cell seems to be pushing in the wall of the other. Since light microscopical observations and physiological studies (Brock, 1961) have shown clearly that both mating partners participate equally in the conjugation act, it is unlikely that sections such as those of Fig. 5 indicate any dominance of one mating type over the other. Rather, it seems likely that accidental cell-wall deformations in the agglutinated clumps, as seen in Fig. 2a, result occasionally in a marked indentation of one cell in contact with another, and further cell-wall deformation during conjugation continues to occur along the path of least resistance. Conjugation has never been seen to be initiated at the site of the bud scar.



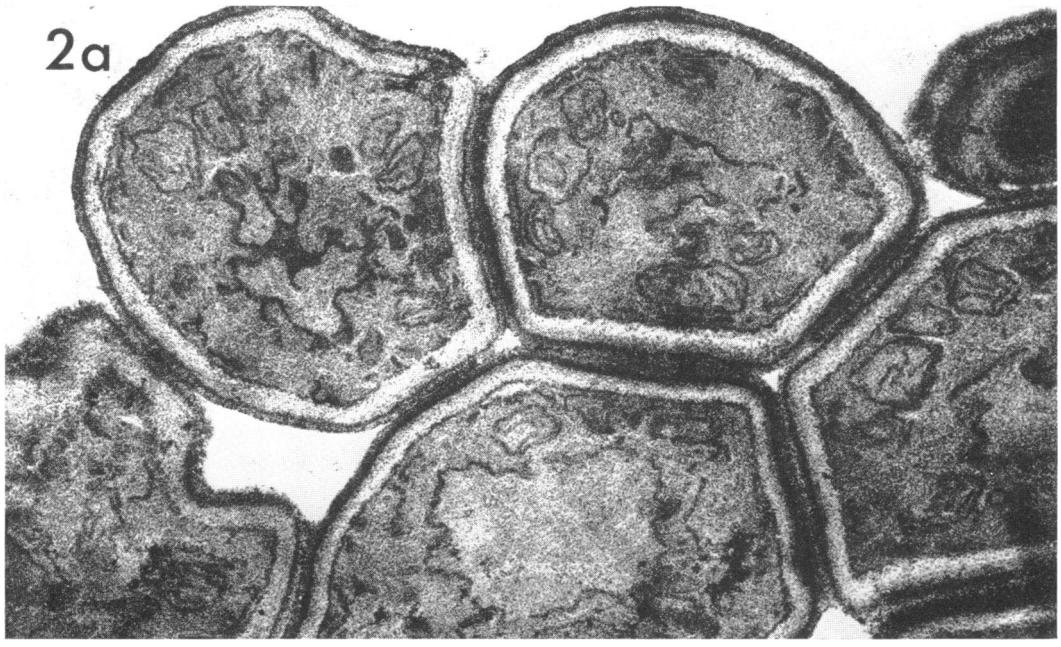
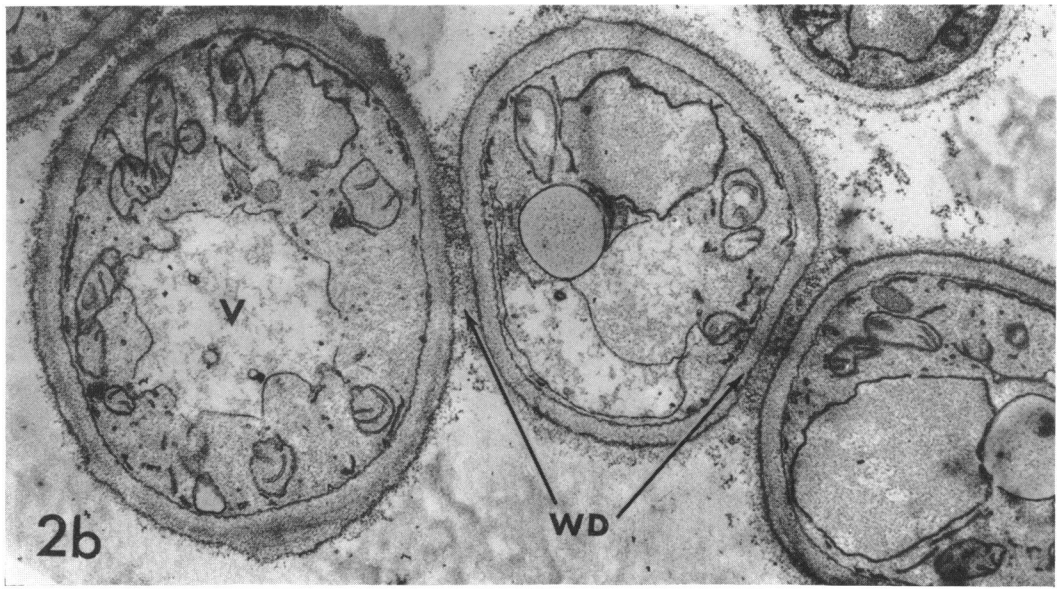


FIG. 1. *General view of a section of Hansenula wingei showing the principal anatomical features. CW = cell wall; N = nucleus; np = nuclear pores; cm = cytoplasmic membrane; M = mitochondria; im = internal membranes.* × *46,000.*

FIG. 2a. *Section through a mass of agglutinated cells, illustrating the intimate contact between the cells and the marked deformation of the cell walls.* × *23,000.*



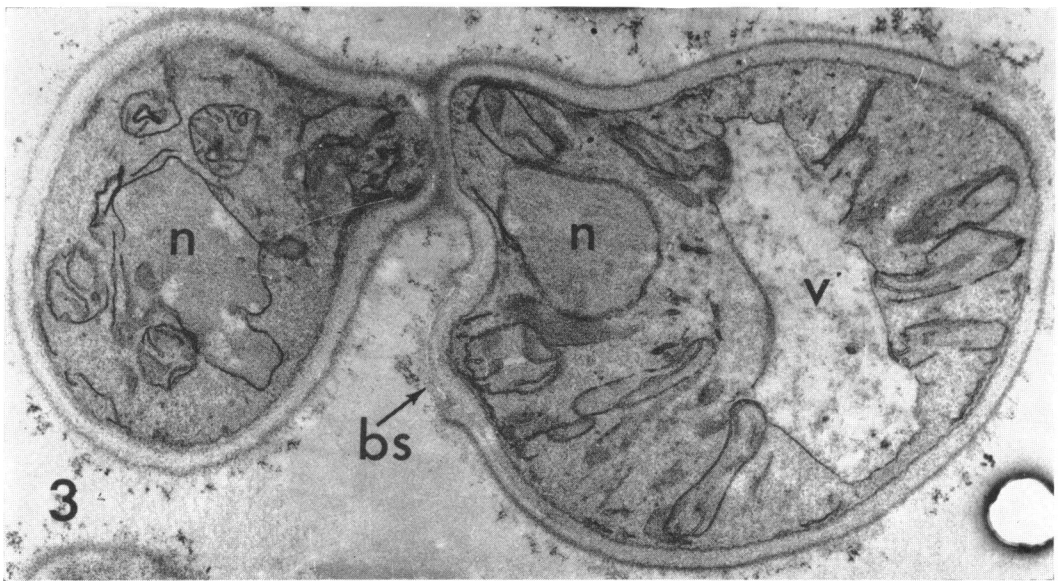
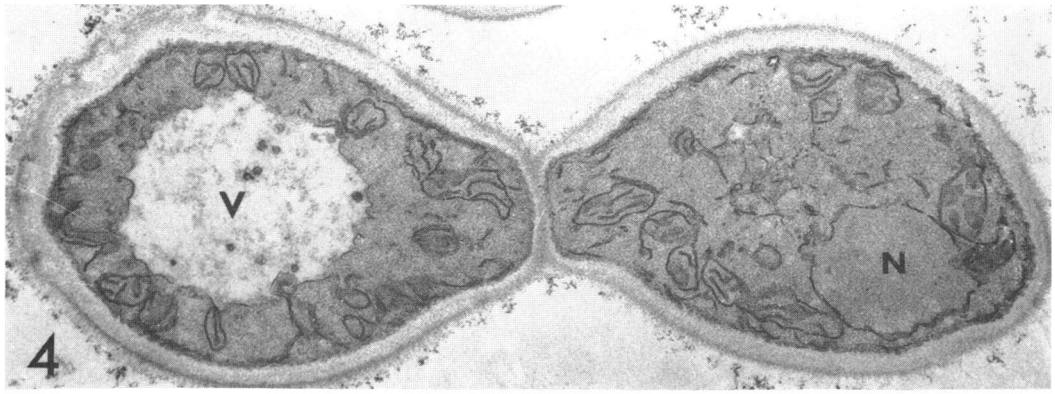
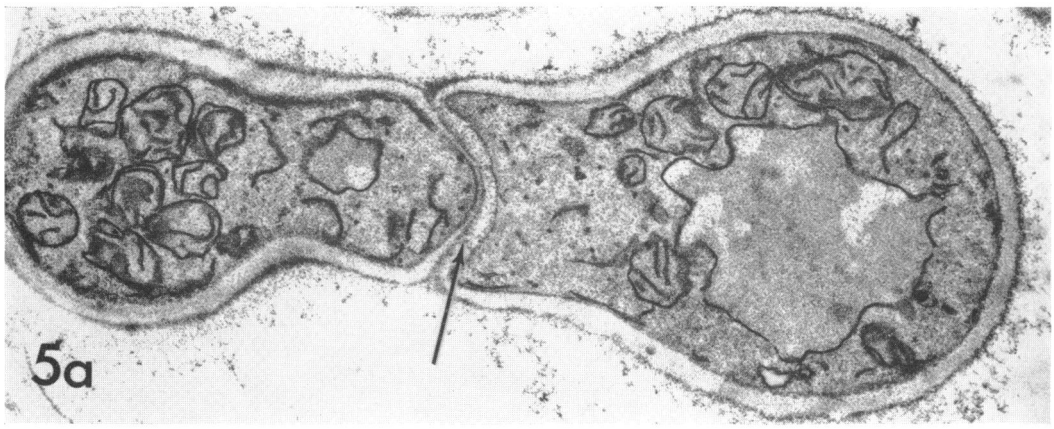


FIG. 2b. *Wall deformation (WD) occurs less drastically when only a few cells of opposite mating type are brought together.* × *14,000.*

FIG. 3. *Section of two cells in an early stage of conjugation. Wall elongation has not taken place along the ovoid axis, causing the cells to appear misshapened. Note the bud scar (bs) near the conjugation tube* × *16,500.*

Cell-wall fusion and dissolution. During conjugation, it is critical that fusion of peripheral portions of the cell wall occur before wall dissolution begins, or else lysis would occur. By deagglutinating cell suspensions at intervals and then examining under the light microscope, it can be shown that fusion takes place between two cells almost as soon as any protuberances are visible (Brock, *unpublished data*). It is difficult to determine from electron micrographs when fusion has occurred, since the events are lost within the electron-dense area in the region of cell contact.





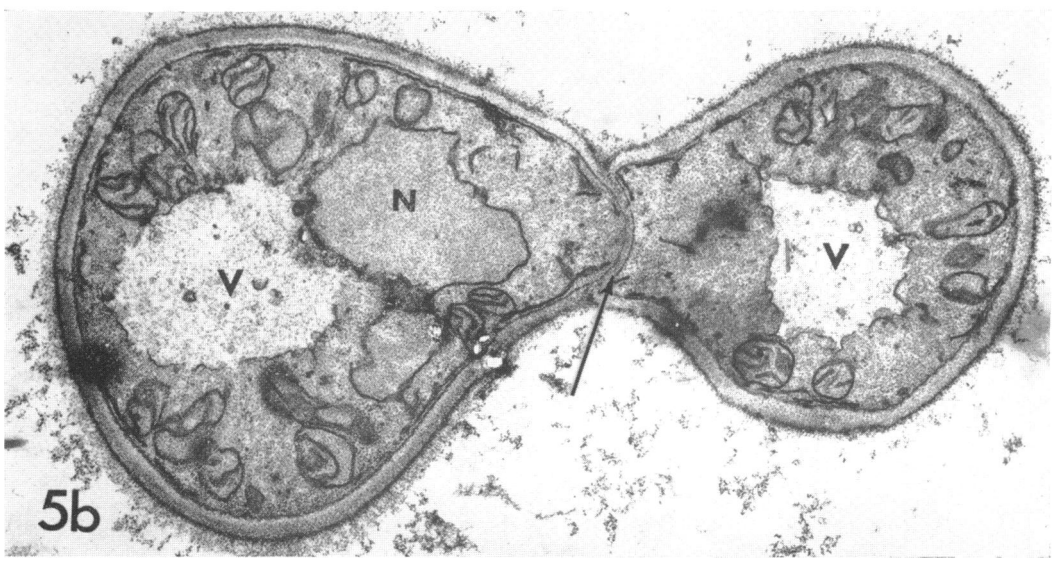
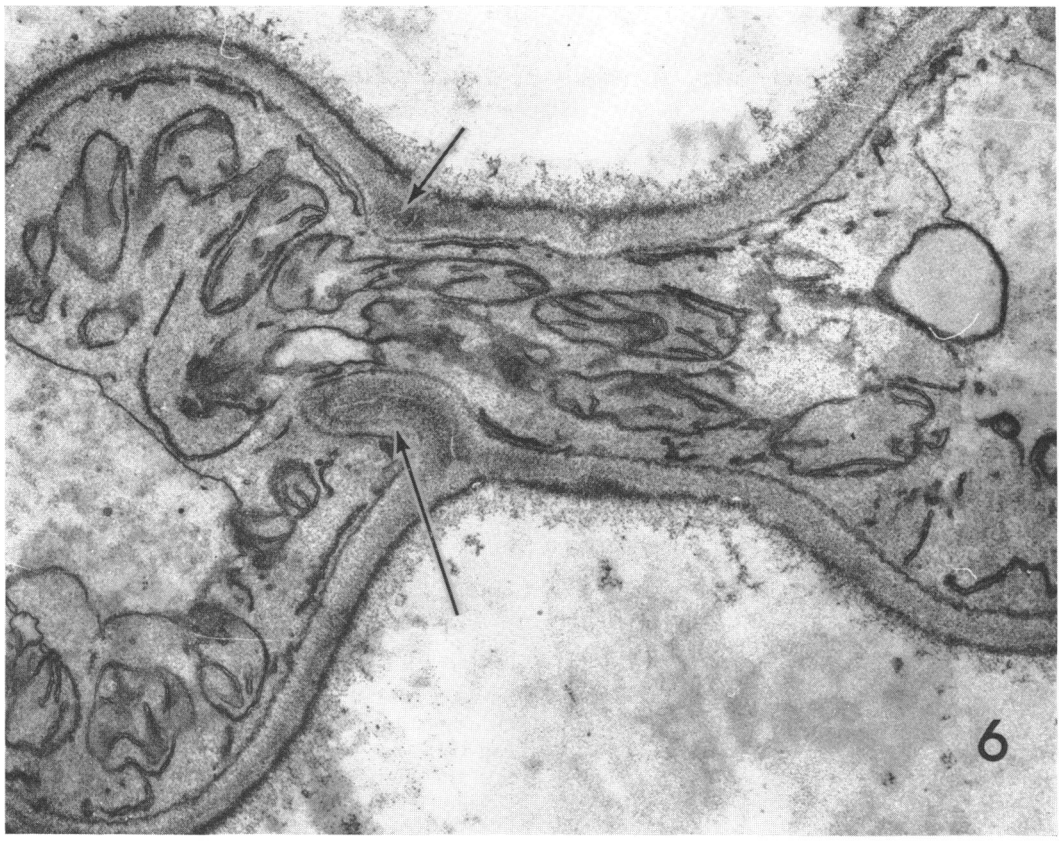


FIG. 4. *Appearance of conjugating cells which have elongated equally along their long axes. V = vacuole.* × *20,000.*

FIG. 5a and 5b. *These micrographs show that in some instances the wall of one of the conjugants appears to be pushing the wall of the other, indicating differences in plasticity of the walls. 5a,* × *19,000; 5b* × *16,500.*



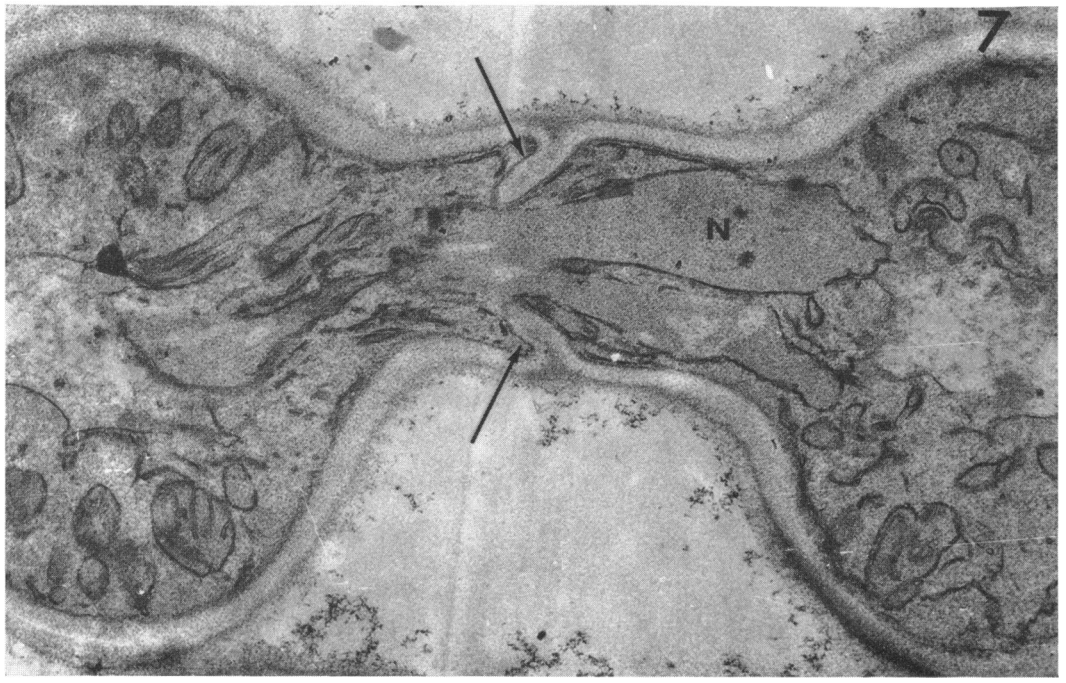


FIG. 6. *Section through the conjugation region showing that a large portion of the cell wall (arrows) remains although cytoplasmic fusion has occurred. The line in the "flap" of the cell wall indicates the junction between the cell walls of the conjugants.* × *24,000.*

FIG. 7. *Section of cells showing that large amounts of cell-wall material remain in the conjugation tube during the latter stages of conjugation. The presence of the nucleus in the tube shows that fusion is occurring or has occurred.* × *23,700.*

Figures 6 and 7, however, reveal stages of wall dissolution in which remnants of fused wall material still exist and provide clues to the earlier history of the fusion process itself. In Fig. 6, the wall has clearly fused completely across the diameter of the conjugation tube, and the line of junction of the two cells can still be seen in the "flap" of wall material, and a trace of this line can be seen in the small protuberance on the opposite side of the conjugation tube, a region which probably represents the erstwhile point of contact of the tip of the flap. Figure 7 reveals cells in which fusion had probably occurred at the center of the conjugation tube, rather than at the periphery. The exact mechanism by which the new cell wall is laid down cannot be inferred from the present material.

In Fig. 6, cell-wall dissolution obviously had begun at a point near the periphery of the conjugation tube, whereas, in Fig. 7, dissolution began from the center. Conceivably, there is no precise control of the location of initial dissolution, and accidental events, such as the way the two cells begin to conjugate, influence this later stage. In gametangial fusion in *Phycomyces blakesleeanus*, Sassen (1962) believed that wall breakdown began at the middle and proceeded centrifugally, although he also saw localized openings in regions distant from the point of dissolution. However, the fusion process in *P. blakesleeanus* differs from that in yeast in that in the former it is occurring in a quite large coenocytic structure, and may not be analogous to cell fusion.

Eventually, all remnants of the cross walls disappear, and the conjugation tube appears to be smooth throughout (Fig. 9 and 10).

Nuclear fusion. In this yeast, nuclei are recognized electron microscopically as large, frequently lobed, membrane-bound regions which are slightly more electron dense than the cytoplasm. In unconjugated cells, only one nucleus is seen per cell. In no case has nuclear division been seen before cell fusion, and in many cases it has been seen that conjugating cells are uninucleate. Since nuclear division occurs only after a bud is formed (Williamson, 1964) and since the cells used in the present work were taken from the stationary phase of growth and do not bud in conjugation medium (Brock, 1961), the nuclei would not be expected to divide.

Nuclear fusion may be initiated even before cell-wall dissolution is complete, and thus, by inference, may begin almost as soon as the cytoplasms of the two cells commingle. This can be seen well in Fig. 8, where it appears as if a lobe of each nucleus has moved towards the center, although extensive cross-wall material remains. Figure 9 shows a situation in which fusion is clearly complete, and the fused nuclear membrane can be traced throughout a great portion of the conjugants. Thus, nuclear fusion probably occurs through interaction of only small regions of the complementary nuclei, and thus does not require the intimate contact of the nuclei throughout most of their surface.

In some of the sections, the nuclei appear to have internal differentiation such as has been reported earlier in other yeast species by Hashimoto et al. (1960) and Thyagarajan, Conti, and Naylor (1962). In some of these electron-transparent regions, fine fibrils (about 20 A in diameter), reminiscent of the appearance of deoxyribonucleic acid fibers, can be seen (Fig. 9 and 10).

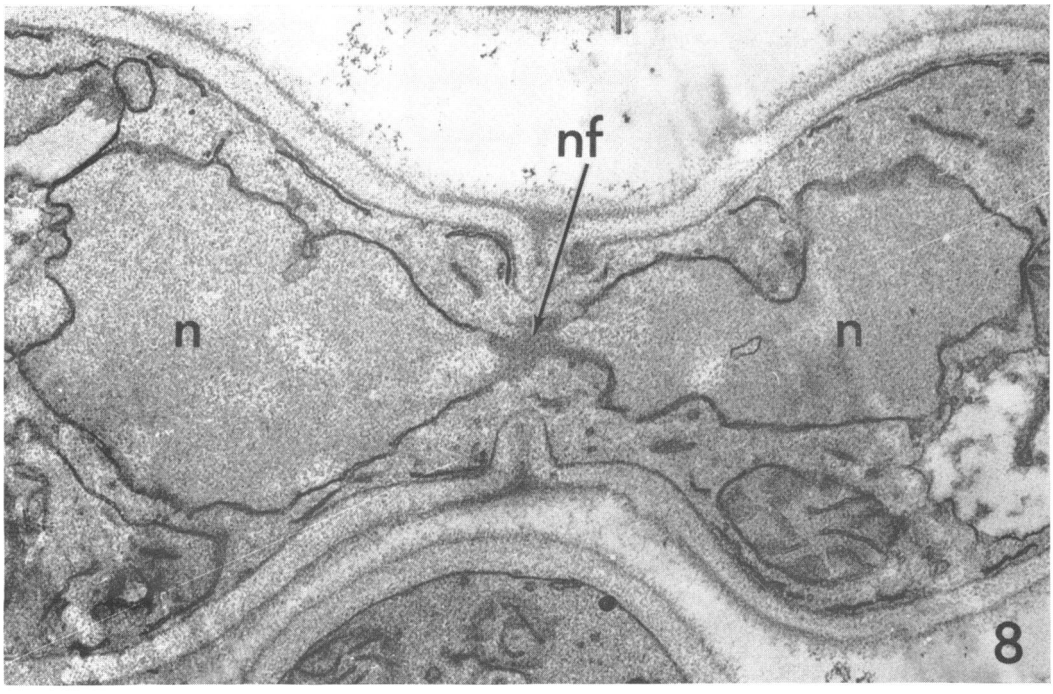
Diploid bud formation. Once nuclear fusion has occurred, the now diploid nucleus apparently contracts to a size nearly that of a haploid nucleus, as shown in Fig. 10. The first diploid bud always develops at right angles to the conjugation tube axis, and approximately in the center of the tube, as shown earlier by light microscopy. This bud usually elongates until it is larger than either of the initial conjugants, and in Fig. 10 it can be seen that the diploid nucleus is moving into this bud while it is still quite small.

Later stages of the process have not been seen in thin sections and indeed are seen only rarely in the light microscope when the present conjugation medium is used. Presumably, after the diploid bud is formed, the remaining cell material of the original conjugating cells eventually autolyzes.

Vacuole development. Vacuoles are rarely seen in unconjugated cells grown under the present conditions (Fig. 1 and 2a), but, as soon as elongation of the conjugation tube begins, vacuoles appear (Fig. 3, 4, 5b, 8, 9, 10). The development of vacuoles during conjugation can also be seen in the light microscope. These vacuoles are membrane-bound, and presumably arise as a consequence of the increase in volume of the conjugating cell without concomitant synthesis of protoplasmic materials.

DISCUSSION

The life history and cytology of yeasts has been reviewed by Winge and Roberts (1958). Conjugation in yeast can occur between two spores, between a spore and a cell, or between two cells. In all cases, the cytological events are similar, beginning with the formation of protuberances which eventually fuse, resulting in a conjugation tube, and an eventual diploid bud at right angles to the tube. Thus, the conjugant shown in Fig. 10 is quite typical of the late stages seen in the conjugation process in a variety of yeasts, so that the structural changes presented in the present paper



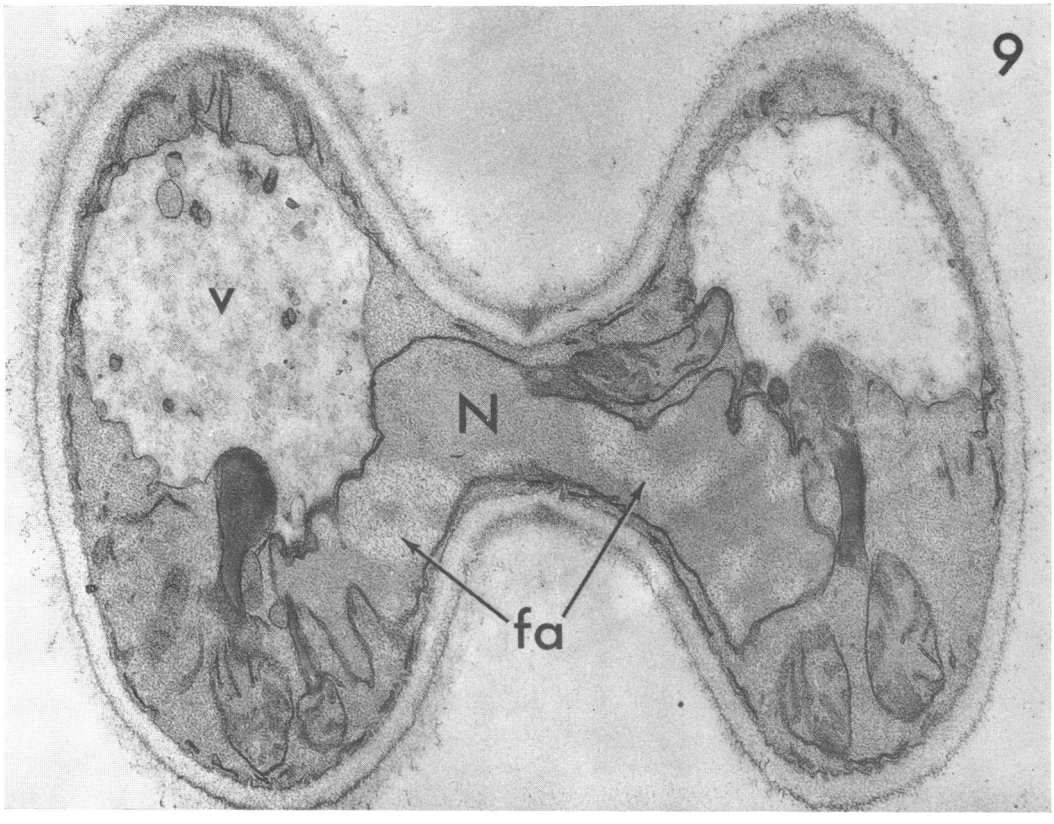


FIG. 8. *Section illustrating what appears to be the fusion of the nuclei.* × *27,000.*

FIG. 9. *Section of cells during a late stage of conjugation; nuclear fusion has been completed. Note the presence of areas of low electron density in the nucleus, which contain fibrils approximately 20 A in width.* × *29,700.*

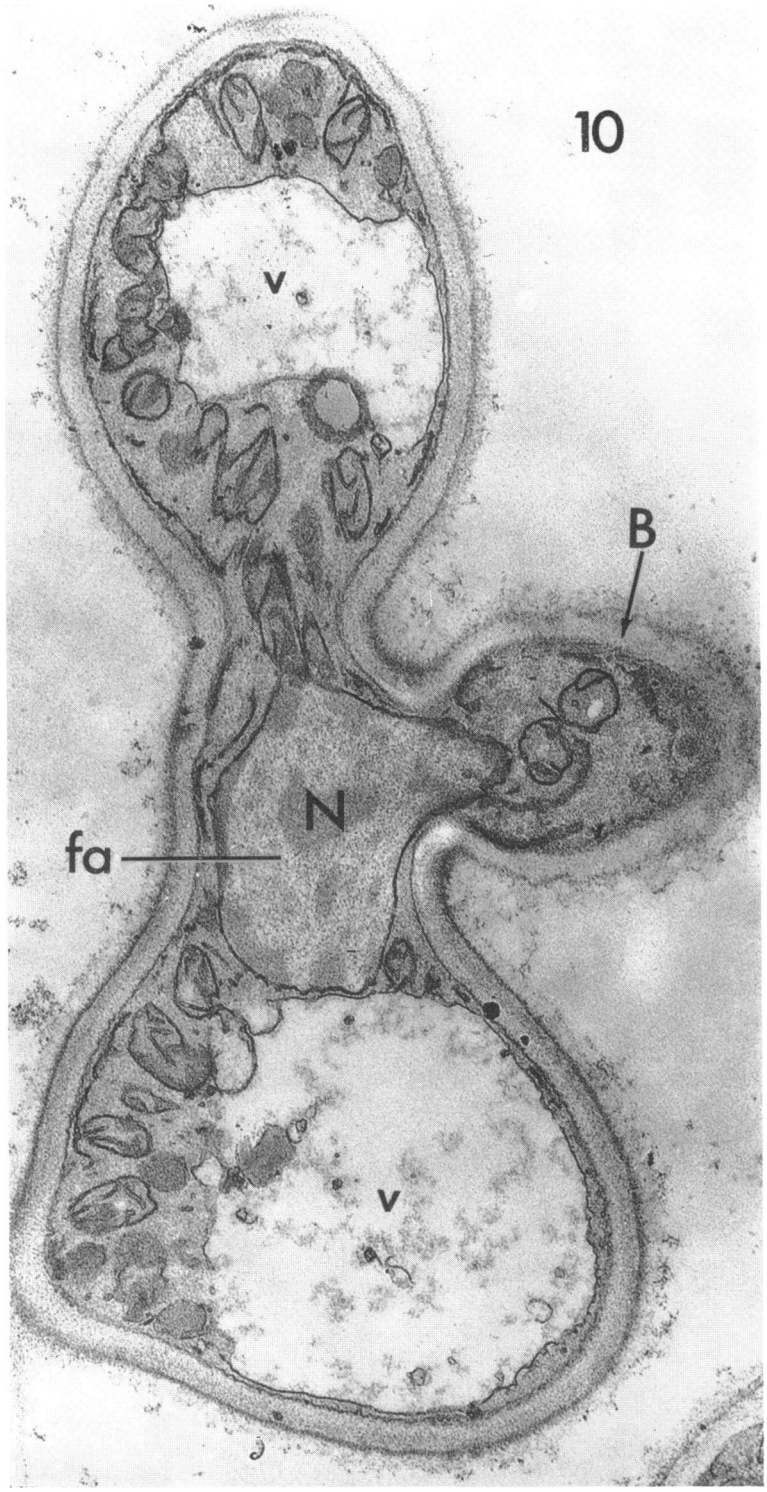


FIG. 10. *Section illustrating the formation of a diploid bud (B) at right angles to the conjugation tube axis.* × *21,000.*

may be presumed to have general relevance. In many yeasts, however, the conjugation process can be initiated between two cells which are not in physical contact, probably as a result of some chemotactic influence (Levi, 1956); such an event has never been observed in *H. wingei*, conjugation occurring only between cells in actual contact (Brock, *unpublished data*). Presumably, the strong agglutination reaction in this yeast eliminates any need for chemotactic attraction, and any diffusible substances which may be involved in conjugation (Brock, 1961) are transferred only after cell contact. However, there is no reason to believe that this modification in the fusion process alters in any significant way the later events of conjugation.

The present results in general confirm the earlier electron microscopical work of Conti and Naylor (1960) on conjugation in *Schizosaccharomyces octosporus*. The high percentage of conjugants formed and the approximate synchrony of the process in *H. wingei* have made the discovery of early stages of the process easier to obtain in the present case, while technical improvements in electron microscopy have made it possible to get more esthetically pleasing pictures; otherwise the results are quite similar. The basic steps in the conjugation process in *H. wingei* have been found to be: deformation of cell wall in the region of contact of two cells, elongation of the cell wall in the area of contact with the formation of protuberances, fusion of cell walls in at least a portion of the contact region, dissolution of the cell wall beginning either from the middle or from one end with the eventual dissolution of all wall fragments in the region of the conjugation tube, migration of the nuclei towards the center and initiation of nuclear fusion even before cell-wall dissolution is complete, fusion of the nuclei by dissolution of a small portion of the nuclear membranes at the point of contact, formation of a diploid bud at right angles to the conjugation tube, and migration of the nucleus into this bud. These latter stages were not seen by Conti and Naylor (1960) because meiosis occurs in *S. octosporus* immediately after conjugation.

Earlier work (Brock, 1961) had shown that protein synthesis was required for the conjugation process, and that both mating types had to participate equally in the process. Current work is underway on the biochemical events of the conjugation process, and the present electron microscopical studies serve to indicate the kinds of biochemical changes which will need to be studied. These include the softening of the cell walls, the synthesis of new cell wall in the region of cell fusion, and the digestion of cell-wall material. Clearly, precise controls must exist so that these processes occur in sequence, and mechanisms must exist to ensure that they occur only in the localized region where two cells are in contact.

As noted in the introduction, the present work may provide a model for fusion processes in other fungi, algae, and higher plants. Undoubtedly, differences in detail exist at both the biochemical and cytological levels, but the overall process in all cases may be similar. Thus, the present work may make it possible for other workers to interpret events of cell fusion in systems in which the process is much rarer and in which it is thus more difficult to obtain a sufficient number of pictures of all stages.

ACKNOWLEDGMENTS

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