STUDIES OF THE FINE STRUCTURE OF MICROORGANISMS

IV. Observations on Budding *Saccharomyces cerevisiae* by Light and Electron Microscopy

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The identification and characterization of the resting and proliferating yeast nucleus are subjects which have been extensively studied by yeast cytologists. Conflicting results have been obtained, and a noticeable lack of general agreement still exists. Differences are particularly apparent with respect to the presence or absence, as well as number, of cytologically demonstrable chromosomes similar in structure to those found in higher organisms. The question as to whether or not a typical mitotic process occurs, comparable to that found in higher organisms, also has not been adequately resolved. In pointing out a few of the difficulties encountered in the investigation of these problems, Mundkur (1954) states that "Incertitude and divergences of opinion of these questions have resulted partly from difficulties inherent in obtaining suitable preparations of organisms so small as yeast for cytological analysis. . . ."

A general review of the pertinent literature reveals that the limited resolving power of the light microscope and lack of suitable staining techniques have been important factors in impeding progress in the field of yeast cytology. The use of the electron microscope to overcome the limitations of the light microscope offers attractive possibilities, and investigations utilizing electron microscopy for the clarification of long standing disputes involving the fine structure of the yeast cell have already yielded promising results. Northcote and Horne (1952) investigated the structure of the yeast cell wall by means of HCl hydrolysis and electron microscopy, whereas Bartholomew and Mittwer (1953, 1954) employed prolonged exposure to ultraviolet light and electron microscopy for the investigation of the budding process and sporulation of yeast. Although the drastic treatment of the cells in the latter study was criticized by Mundkur (1954), both of these studies helped to establish the value of the electron microscope to the field of yeast cytology.

The development of ultrathin sectioning techniques, and their application to the study of the fine structure of the yeast cell, also proved to be quite revealing (Bartholomew and Levin, 1955; Agar and Douglas, 1955, 1957). Although the studies of Agar and Douglas (1957) uncovered many details of the yeast cell structure, technical difficulties confined their work to a study of the resting vegetative cell. The development of an improved ultrathin sectioning technique (Hashimoto and Naylor, 1958) enables one not only to observe the structure of the yeast ascospore (Hashimoto *et al.*, 1958*b*) but also to study the growing and dividing yeast cell (Hashimoto *et al.*, 1958*c*). In the present study, this technique was utilized for the investigation of the behavior and structure of the yeast nucleus during bud formation. Similar observations were also carried out on other cellular components. In addition to the electron microscopy of the sectioned yeast cell, conventional light microscopical methods were employed. These parallel studies established a basis for interpretation and comparison of observations made from light and electron micrographs.

MATERIALS AND METHODS

Yeast. A distillers yeast, *Saccharomyces cerevisiae* strain M-1, was employed for all studies. Ascospore structure and mode of germination of this yeast have been described previously (Hashimoto *et al.*, 1958*a*, *b*). 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; pH 4.8) for 3 days at 30 C. The

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cells were then harvested aseptically and transferred to a liquid medium (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; pH 6.0). The cells were kept overnight at 4 C, then incubated with vigorous shaking at 23 C. Under the experimental conditions employed, a degree of synchronization of cell growth was obtained which was satisfactory for electron microscopical investigations.

Specimen preparation. Aliquots of the culture were taken at appropriate intervals for study by light microscopy, and preparation of ultrathin sections. The technique for the preparation of ultrathin sections was as described by Hashimoto and Naylor (1958) except for the substitution of potassium permanganate for osmium tetroxide as the fixing agent. The usefulness of potassium permanganate as a fixative for animal cells was suggested earlier by Luft (1956). Potassium permanganate has also been found to be a satisfactory fixative for the study of vaccinia virus (Epstein, 1958), *S. cerevisiae* ascospores and vegetative cells, (Hashimoto *et al.*, 1958*b*, *c*), and *Escherichia coli* (Mercer, 1958) by means of ultrathin sectioning and electron microscopy. In the present investigation it was found that treatment of the cells with a 1.5 per cent aqueous solution of potassium permanganate for 30 min at 23 C resulted in satisfactory fixation. Subsequent treatment of the cells was as described by Hashimoto and Naylor (1958). Here again, prolonged treatment with partially polymerized *n*-butyl methacrylate was found to be highly effective in minimizing explosion of the cells during polymerization. Sections were cut with a Porter-Blum microtome equipped with a glass knife. Occasional preparations were made employing formvar, rather than collodion, as the grid substrate. An RCA EMU-2b electron microscope, equipped with a 50 μ limiting objective aperture was used for all electron microscopic observations.

Cytochemical techniques and light microscopy: In addition to the cytochemical methods described in a previous study (Hashimoto *et al.*, 1958*b*) a modification of Lindegren's Carnoy-perchloric acid-Giemsa staining technique (Lindegren *et al.*, 1956) was employed for staining of the nucleus. Contrast between chromatin and cytoplasm was greatest when the cells were washed twice with 0.067 M phosphate buffer after hydrolysis. Cells were stained with an aqueous Giemsa solution (12 drops in 10 ml of distilled water at room temperature) in a test tube. The stained cells were directly mounted under a cover slip onto a clean slide and the edges sealed with vaspar. Light micrographs were taken with Kodak microfile film. Occasionally a green filter was used to increase the contrast in the micrographs.

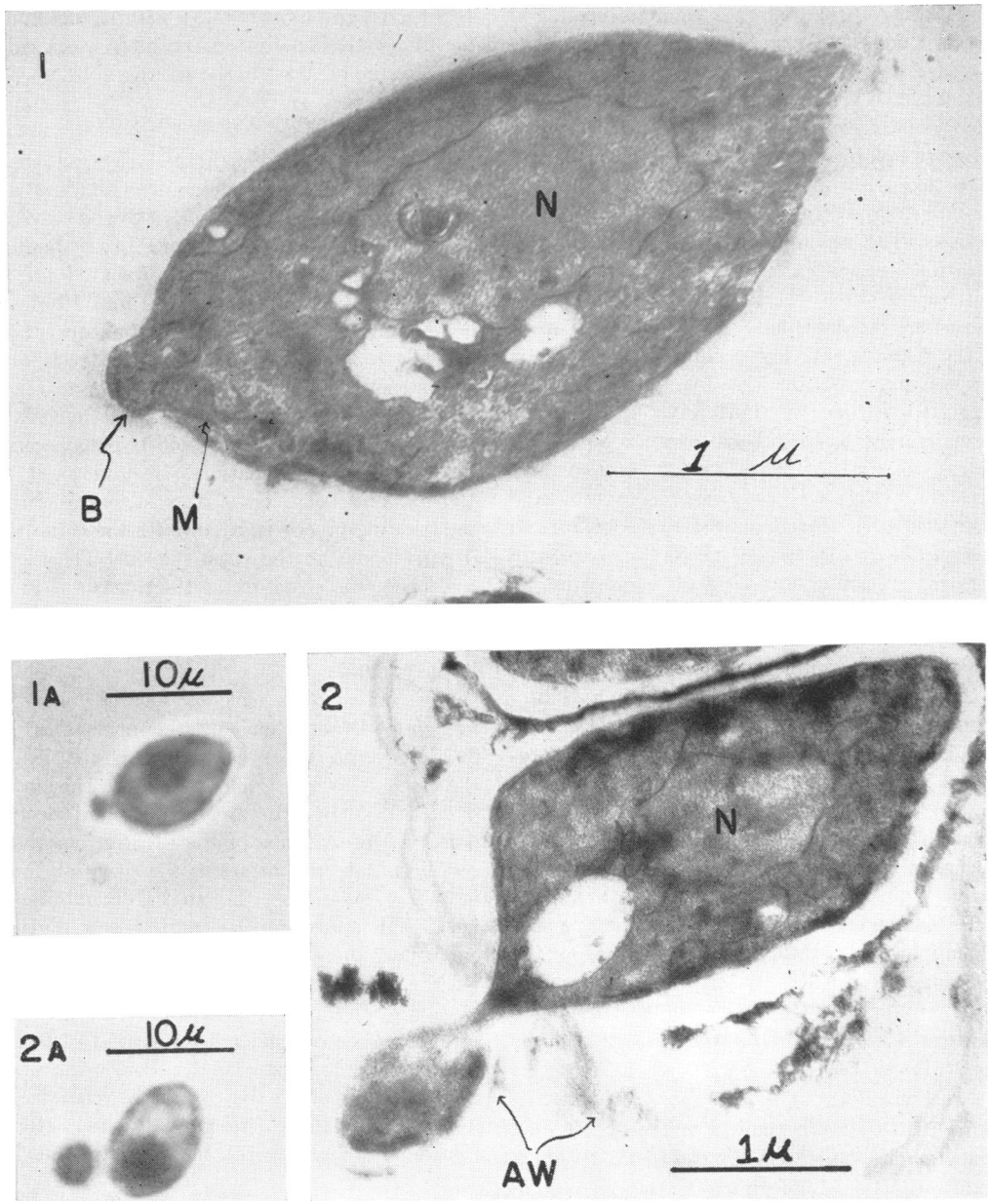
RESULTS AND DISCUSSION

Figures 1 to 6 are light and electron micrographs of *S. cerevisiae* undergoing budding with concomitant nuclear division, arranged to represent a logical sequence of stages in the budding process. Figures 7 to 10 are presented for the purpose of discussion. Figures 9 and 10 clearly demonstrate the highly lobulate nature of the dividing nucleus as revealed by serial sectioning. The structure labeled N[2] in the electron micrographs is considered to be the yeast nucleus on the basis of previous studies of its behavior during ascospore germination (Hashimoto *et al.*, 1958*b*). The behavior of this structure during the budding process substantiates the claim that it corresponds to the yeast nucleus. The identification of the structure as the nucleus is in accord with the results of other investigators (DeLamater, 1950; Mundkur, 1954; Agar and Douglas, 1957).

Structure and behavior of the nucleus during bud formation. Observations on the nucleus of the budding yeast cell reveal that it is identical to the nucleus of the yeast ascospore (Hashimoto *et al.*, 1958*b*) in structure, texture, and electron density. Observations of the dividing yeast cell revealed that, in contrast to the situation found in higher organisms, the nuclear membrane of yeast did not disappear during cell division, confirming the observations of Mundkur (1954). On the basis of light microscopic studies the retention of the nuclear membrane during cell division has been considered for years to be characteristic of the ascomycetes, a conclusion which is now confirmed in the case of *S. cerevisiae* by means of electron microscopy. Previous studies (Hashimoto *et al.*, 1958*c*), indicated that nuclear

[2] The following abbreviations are used in the illustration of electron micrographs: *AW*, ascus wall; *B*, bud; *CW*, cell wall; *CM*, cytoplasmic membrane; *CP*, constriction point of the nucleus; *L*, lipoidal inclusion; *M*, mitochondrialike bodies; *N*, nucleus; and *V*, central vacuole.

division is essentially an autonomous process in that nuclear separation is not due to pressure exerted by the constricting cell wall. Figures 3, 4, and 5 also demonstrate that nuclear constriction and separation is not due to the constriction and pinching off of the nucleus by the cell wall. When the bud approaches the size of the mother cell, a sharp wedgelike constriction forms in the



Figures 1, 1A, 2, and 2A. These electron micrographs, together with the corresponding light micrographs show the initiation of bud formation within a yeast cell. The nucleus appears to retain its central position within the cell at this stage. Note the concentration of mitochondrialike bodies at the site of bud formation. Figure 2 shows a germinating ascospore initiating bud formation. All light micrographs are of cells stained by the modified Carnoy-perchloric acid-Giemsa staining procedure described in the text.

nuclear membrane. This constricted portion of the nucleus appears to be displaced in the direction of the mother cell. The constriction then deepens as seen in figures 3 to 5, eventually resulting in complete separation of the mother nucleus into two morphologically identical structures. Cell wall formation and separation of the bud and mother cell then follows.

Centriole. Studies by DeLamater (1950), Bartholomew and Mittwer (1953), and Mundkur (1954) indicate that a centriole is involved in the orientation of the dividing nucleus. A structure corresponding to the centriole described by these investigators was not observed in any of the electron micrographs. Our failure to observe the presence of this structure may be due to the threadlike nature of this organelle and thinness of the sections, which minimizes the possibility of obtaining a plane of sectioning through the longitudinal axis of this structure. The electron micrographs obtained to date are insufficient for substantiation or negation of the presence of a centriole in the dividing yeast cell.

Cell wall and cytoplasmic membrane. Actively growing and dividing yeast cells, in common with germinating ascospores (Hashimoto *et al.*, 1958*b*) were found to have a thin cell wall of relatively low electron density. In electron micrographs the cell wall appeared non-electron dense and was clearly visible only after it had been stained with lanthanum nitrate (Hashimoto *et al.*, 1958*b*) or overexposed during photographic enlargement (figure 7).

The cytoplasmic membrane was found to be smooth and closely adherent to the cell wall. This is in strong contrast to the invaginated structure of the cytoplasmic membrane found in resting yeast cells (Agar and Douglas, 1957). This can be attributed to differences in technique, as well as to differences in the physiological state of the cells studied.

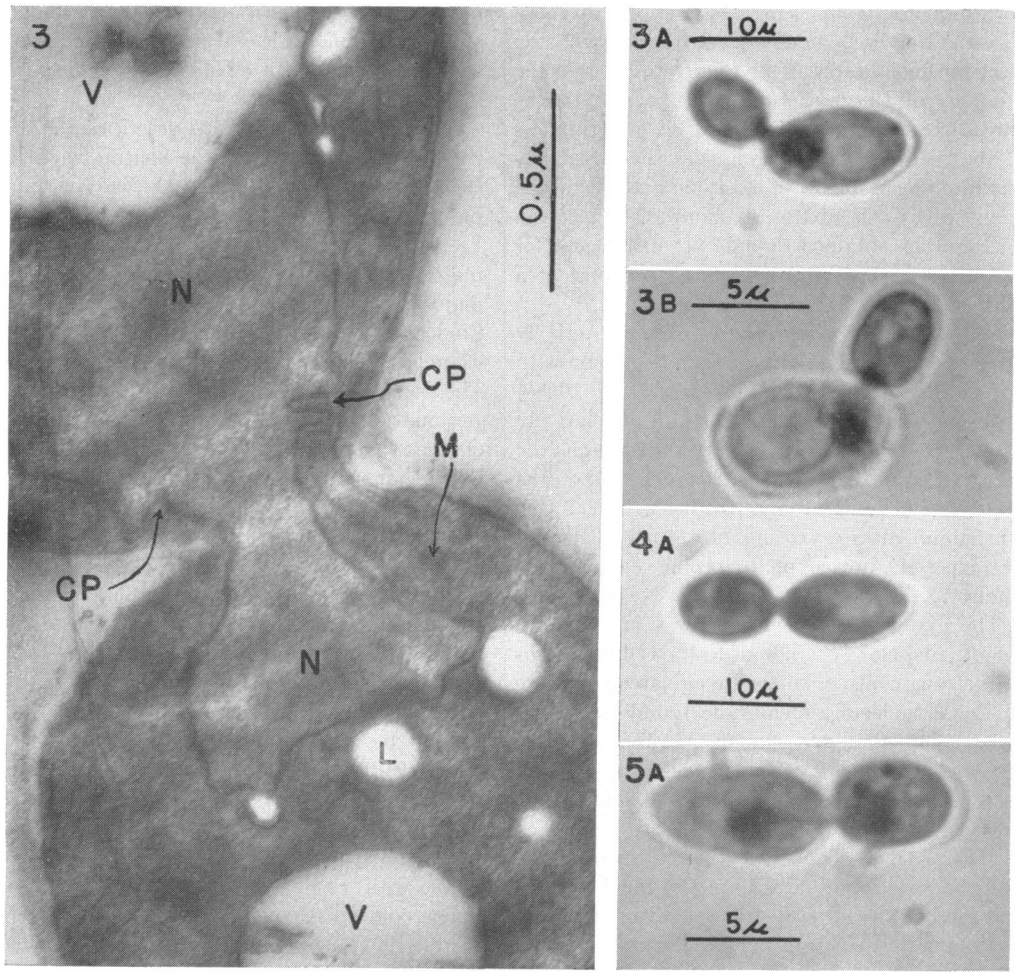
Mitochondria. The presence and structure of mitochondria in yeast, a subject of much dispute and interest, was first demonstrated by ultrathin sectioning and electron microscopy by Agar and Douglas (1957). They found that most of the mitochondria were at the periphery of the cell. This is also apparent from our electron micrographs. It must be mentioned, however, that in spite of the vigorous aeration of the cells during growth, the number of *cristae mitochondriales* was surprisingly small (*M* in figure 8), and in some cases their presence was questionable (*M* in figure 3). Although yeast mitochondria are structurally and physiologically similar to the mitochondria of higher organisms, the small number of cristae may be a characteristic of yeast mitochondria. The structure (*M* in figure 3) lying adjacent to the nucleus at the site of budding may well be a mitochondrion migrating from the mother cell into the bud, but the absence of definite cristae makes this interpretation uncertain. Similarly, the structures labeled *M* in figure 1 may be mitochondria accumulating at the site of bud formation, presumably a site of increased physiological activity. The filamentous structures found in the yeast cell and considered as mitochondria by Müller (1958) were not observed.

Central vacuole. A detailed description and discussion of the nature of the central vacuole of the yeast cell can be found in various earlier publications (DeLamater, 1950; Lindegren, 1949; Lindegren *et al.*, 1956; Mundkur, 1954; Hashimoto *et al.*, 1958*b*; Agar and Douglas, 1957). The majority of yeast cytologists, in contrast to Lindegren, support the view that the central vacuole is not an integral part of the nucleus. The behavior of this structure during ascospore germination (Hashimoto *et al.*, 1958*b*), separation of the vacuole from the nucleus by definite limiting membranes as shown in figure 6, and its behavior during the budding process gives additional support to the claim that it is not an integral part of the nucleus.

Regardless of differences in the interpretation of the light micrographs, the usefulness of the modified Carnoy-perchloric acid-Giemsa staining technique was evident throughout this study. Although chemical treatment of the yeast cell usually results in distortion or collapse of the large central vacuole (Mundkur, 1954), this technique minimizes these undesirable effects. McClary (1958) demonstrated the specificity of this staining technique by correlating results obtained with it and with a modified Feulgen stain under controlled conditions. In addition Townsend (1957) whose micrographs, particularly figures 5 and 6, are quite similar to those obtained in the present study, demonstrated that only the body lying adjacent to the central vacuole was Feulgen positive in the macro yeast cell. It must be pointed out, however, that the Carnoy-perchloric acid-Giemsa staining technique frequently produced nonspecific colorization of cytoplasmic granules unless precautions were taken

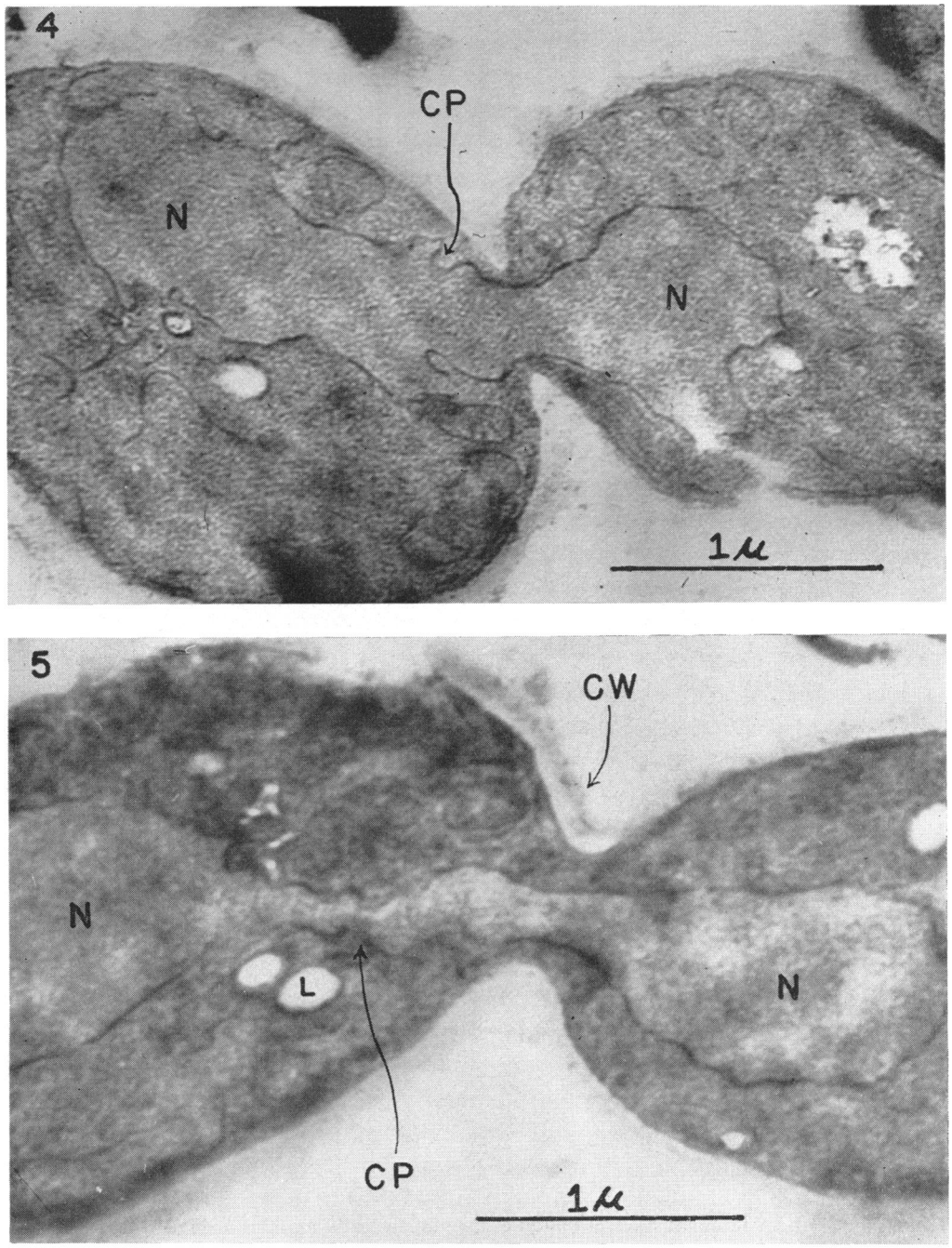
to avoid insufficient hydrolysis. Considering the difficulties encountered by many previous investigators (Nagel, 1946; Levan, 1947; Lietz, 1951) in staining the yeast nucleus, it is clear that this technique can be regarded as an important technical advancement.

Use of the modified Carnoy-perchloric acid-Giemsa staining procedure usually resulted in uniform staining of the nondividing yeast nucleus. On the other hand, it was found that when actively growing and dividing yeast cells were stained, areas with different color intensities were apparent within the nucleus. In general, from 1 to 3 areas within the nucleus appeared to be more deeply stained than the rest of the nucleoplasm. These deeply stained areas have been considered to be chromosomes, morphologically comparable to those found in higher organisms, by a number of workers. In contrast to this, Mundkur (1954) considers these to be aggregations of chromatin particles produced by the procedures involved in fixation, hydrolysis, and



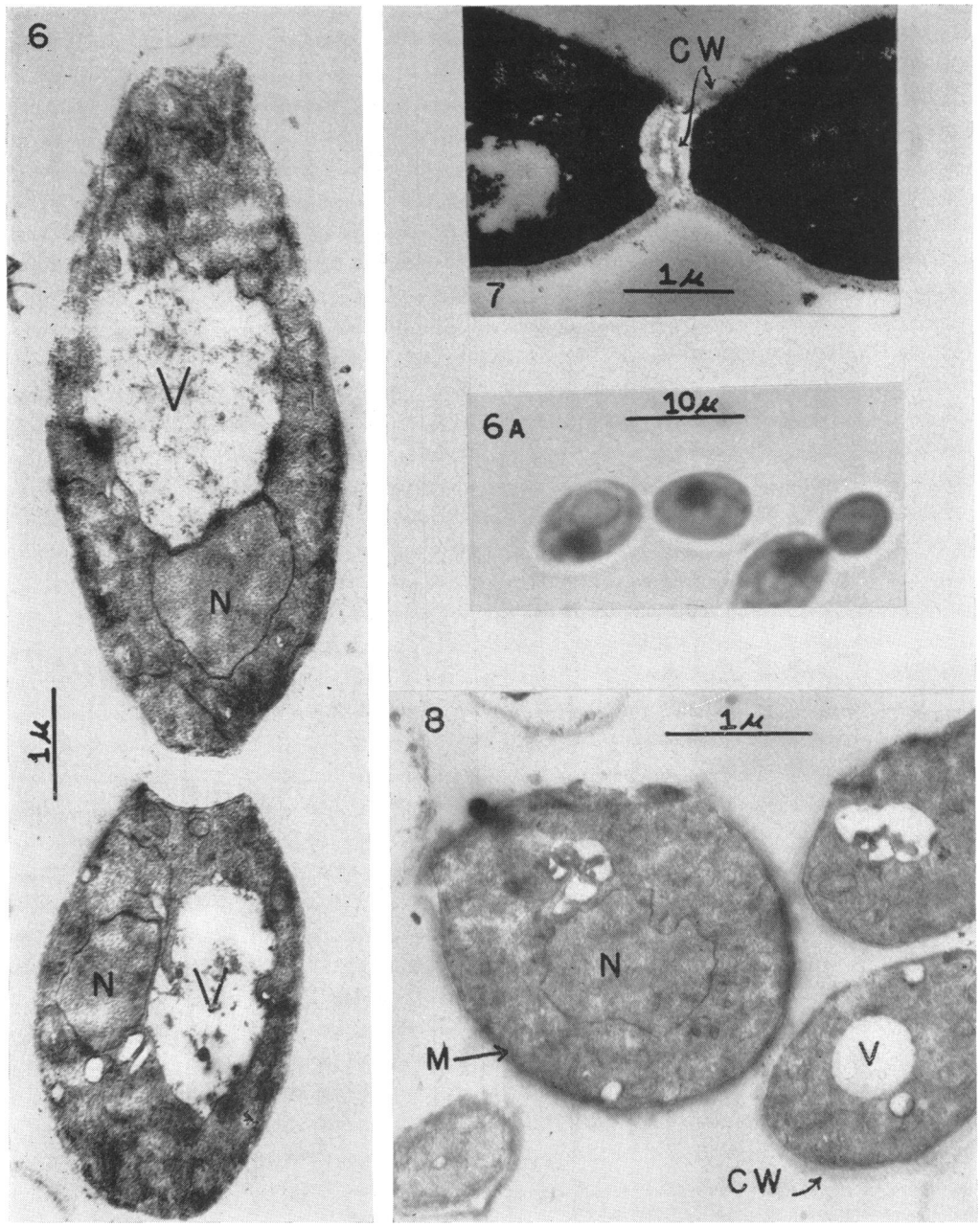
Figures 3, 3A, 3B, 4, and 4A. Intermediate stages of cell division. Note the migration of a portion of the nucleus into the bud, and the constriction of the nuclear membrane which is displaced in the direction of the mother cell. This constriction is not evident in any of the light micrographs. Figures 3*A* and 3*B* illustrate the differential staining of the nucleus which may be erroneously interpreted as chromosomal figures.

Figures 5 and 5A. Electron and light micrographs of yeast cells in a well advanced stage of budding. Note that the nuclei are still not separated, and that the nuclear membrane is intact.



Figures 4–5

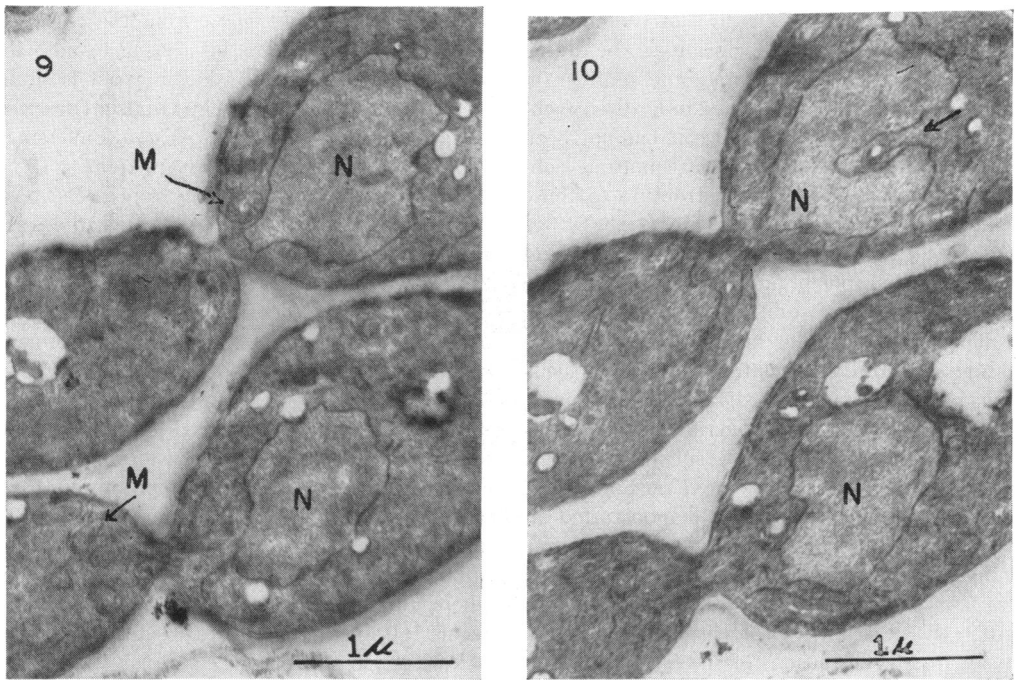
staining. Other investigators (Beams *et al.*, 1940) also consider the nucleus to be a uniformly staining body, and conclude that the division of the yeast nucleus involves an amitotic process. This conclusion has been criticized by Mundkur (1954) as being unwarranted in the light of genetic evidence. However, this criticism is not completely justifiable since amitosis has been used to describe the cytological process of direct nuclear division without the emergence of typical chromo-



Figures 6 and 6A. A terminal stage of budding. Each of the cells contains a single nucleus, although the cells are still connected by a common cell wall. Note the displacement of the nuclei from the site of bud formation towards the center of the cell.

Figure 7. A portion of figure 6 overexposed to more clearly illustrate the presence of the connecting cell wall.

Figure 8. An electron micrograph of an actively growing yeast cell showing a typical mitochondrion, containing short, but definite *cristae mitochondriales*. Note the partially collapsed vacuole adjacent to the nucleus.



Figures 9 and 10. Parallel sections of the same yeast cells illustrating the lobulate structure of the nucleus. Note the invagination of the nucleus (*arrow*, figure 10) which is absent from the more peripheral, parallel section of the cell shown in figure 9.

somes or a spindle apparatus (Robinow, 1956). Since Beams *et al.* (1940) investigated the division of the yeast nucleus by conventional light microscopic techniques, the existence of uniformly dispersed submicroscopic chromatin particles, as postulated by Mundkur (1954), is not excluded. The lack of effect of colchicine treatment (Beams *et al.*, 1940) may be characteristic of the chromatin dispersed within the yeast nucleus.

Although Beams *et al.* (1940) and Mundkur (1954) failed to detect structures corresponding to chromosomes within the yeast nucleus, other investigators have reported the clear-cut demonstration of chomosomes by use of various staining techniques. DeLamater (1950) demonstrated a maximum of 4 chromosomes within the yeast nucleus; Winge (1951), 2 to 4; Lietz (1951), 3; and Subramaniam (1952), 2. These observations are somewhat difficult to reconcile with genetic evidence (Lindegren, 1949) indicating that at least 4 chromosomes are present in haploid cells of *S. cerevisiae*. The differences obtained with respect to the normal chromosome complement of the haploid yeast cells clearly reflect the limitations of the staining procedures employed for chromosome demonstration. Although the presence of these chromosomes may be due solely to artifacts produced during the staining process (Mundkur, 1954), other factors may be involved. As seen in figures 9 and 10, the dividing yeast nucleus is a highly lobulate structure. In the light of this observation it seems not too surprising to observe areas with different color intensities within the stained nucleus. Irregular staining may be the result of the lobulate nature of the nucleus, combined with the aggregation of chromatin particles and collapse of the central vacuole caused by the chemical procedures involved in the staining process. The artifacts which can be produced by the various staining techniques, and the assumption that yeast nuclei must contain structures completely analogous to the chromosomes found in higher organisms, may have misled some yeast cytologists. We are in complete agreement with the statement of Ganesan and Swaminathan (1958) that, "Until a standardized technique is evolved which will

give consistently similar pictures of the chromosomes in many cells, no conclusion can be drawn concerning the correct chromosome number in yeast." The development of a standardized technique may not be possible since it has not been clearly established that the yeast nucleus contains chromosomes similar in structure to those found in higher organisms.

A critical examination of all the light and electron micrographs obtained to date favors the view that structural units, comparable to the chromosomes found in higher organisms, are lacking. The homogeneous texture of the nucleus during various stages of division, and during various stages of ascospore germination (Hashimoto *et al.*, 1958*b*) tends to substantiate this conclusion. It should be pointed out, however, that chromosomes have been demonstrated in electron micrographs of sectioned cells of higher organisms (Borysko, 1953; DeRobertis, 1956; Fawcett, 1956), and that failure to demonstrate them in the yeast nucleus by electron microscopy may be due to limitations of the techniques employed. The efforts of Gibbons and Bradfield (1956), and Moses (1956) to develop techniques for elucidating the complex fine structure of chromatin in nuclei are particularly significant. Further application and modifications of these techniques may enable one to observe the exact nature of chromatin structure in the yeast nucleus.

From these observations, and in the light of other studies on the yeast nucleus, it seems most logical to accept the concept that submicroscopic units of chromatin, functioning as chromosomes, are dispersed throughout the nucleus. Although genetic studies (Lindegren, 1949) clearly demonstrate that mitosis occurs in yeast, the cytological evidence is far from convincing. This situation is also paralleled in the field of bacteriology and protozoology. Robinow (1956), in discussing the amitotic process considered to be occurring in the macronuclei of ciliated protozoa, points out that, "It is thus certain that the macronucleus arises from chromosomes even though they are not individually discernible either during or between divisions in the full grown organ. It is difficult to imagine how duplicates of all the individuals of a set of chromosomes can be equitably distributed between the halves of a directly dividing macronucleus. That it is somehow done with considerable, if not absolute, efficiency is evident from the researches of Sonneborn. . . ." It is interesting to note that the mode of nuclear division in yeast is similar to the process of direct nuclear division described in various fungi (Robinow, 1957*a*, *b*; Bakerspigel, 1958) although differences are apparent.

For the sake of clarity we propose the terms "cryptomitosis" and "cryptomitotic" to describe the existing situation in yeast cytology where there is a genetic basis without an adequate cytological basis for the establishment of the occurrence of the mitotic process. The proposal of this definition seems to be warranted in view of the differences between Mundkur (1954), and Beams *et al.* (1940) which seem to be due mainly to ambiguous terminology, and an inappropriate choice of terms. The term "cryptomitosis" may also be profitably employed in describing the unique mode of nuclear division found so ubiquitously in other protists, such as bacteria, where functionally perfect mitosis appears to occur, although cytological substantiation is lacking or inconsistent.

Although the processes of budding and ascospore germination (Hashimoto *et al.*, 1958*b*) have been described, detailed studies on the process of sporulation in *S. cerevisiae* appear to be lacking. The mechanism and details of the sporulation process in yeast are currently under investigation.

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SUMMARY

The budding process of *Saccharomyces cerevisiae* was followed by means of electron microscopy of thin sections and conventional light microscopical techniques, combined with a nuclear staining method. The actively dividing nucleus was found to be highly lobulate in shape and homogeneous in texture. The nuclear membrane was observed to persist throughout the various stages of nuclear division. Structures identifiable as chromosomes were not observed in any of the electron micrographs.

The available evidence indicates that sub-

microscopic units of chromatin are dispersed throughout the yeast nucleus. The term "cryptomitosis" is proposed to describe the situation in which genetic evidence indicates the occurrence of mitosis, without an adequate or consistent cytological basis. Structure and behavior of various components of the yeast cell during the budding process were also described.

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