

ELECTRON MICROSCOPY OF *RHODOTORULA GLUTINIS*

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ABSTRACT

THYAGARAJAN, T. R. (Dartmouth Medical School, Hanover, N. H.), S. F. CONTI, AND H. B. NAYLOR. Electron microscopy of *Rhodotorula glutinis*. *J. Bacteriol.* **83**:381-394. 1962.—The structure and manner of nuclear division in *Rhodotorula glutinis* was studied by electron microscopy of ultrathin sections. Parallel studies with the light microscope, employing conventional staining techniques and phase-contrast microscope observations on nuclei in living cells, were carried out.

The nucleus is spherical to oval and is bounded by a nuclear membrane. Intranuclear structures, identified as nucleoli, and electron-transparent areas were observed. The nuclear membrane persists throughout the various stages of cell division. Observations of the nucleus with the electron microscope revealed that nuclear division occurs by a process of elongation and constriction similar to that seen in both living and stained cells.

The fine structure of mitochondria and other components of the yeast cell and their behavior during cell division are described. The absence of vacuoles in actively dividing cells of *Rhodotorula glutinis* lends further support to the view that the vacuole is not an integral part of the nucleus. The results with the electron microscope generally support and considerably extend those obtained with living and stained cells.

Although disagreement still persists as to the structure of the yeast nucleus and the mode of nuclear behavior during vegetative cell division, recent studies of ultrathin sections of yeast with the electron microscope have proved to be valuable in solving some of the earlier conflicting reports. The presence of a nuclear membrane and its persistence throughout the division

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process has been established for some yeasts (Hashimoto, Conti, and Naylor, 1958, 1959; Conti and Naylor, 1959, 1960a). Although no structural details were observed in the nuclei of vegetative cells, differentiation within the nucleus of *Saccharomyces cerevisiae* was noticed during sporulation (Hashimoto et al., 1960). Yotsuyanagi (1960) reported the presence of areas of low electron density, which he considered to be chromosomes, within the nucleus of the vegetative yeast cell.

A series of studies on the living and stained nuclei of many strains and species of yeast (Subramaniam et al., 1959; Royan and Subramaniam, 1960; Thyagarajan, 1959, 1961) revealed within the nuclei structures which were resolvable into chromocenters and nucleolar equivalents. Recently, Robinow (1961) presented evidence for mitosis in *Lipomyces lipofer*. A nucleolus was also observed in this yeast, thus confirming the earlier report of Ganesan and Roberts (1959).

A previous study showed that it is possible to observe with the phase-contrast microscope the nuclei in living cells of *Rhodotorula glutinis* when the cells are grown in a dilute barley malt wort medium (Thyagarajan and Naylor, 1961). A study of this yeast, correlating observations with both light and electron microscopes, was carried out in the hope that our knowledge of the cytology of yeast could be further expanded.

MATERIALS AND METHODS

R. glutinis, supplied by L. J. Wickerham, was employed for all investigations. Cells were routinely grown in barley malt wort medium of specific gravity 1.005, adjusted to pH 4.6. Although growth was slow, this medium was preferred because large fat bodies, which mask the nucleus of cells grown in media containing glucose, peptone, and yeast extract, did not accumulate during the early stages of growth. To obtain enough material for fixation, a 48-hr

test tube culture was transferred to a Roux flask containing 150 ml of barley malt wort medium and was incubated for 48 hr at room temperature. Under the conditions employed, a degree of synchronization of cell growth was obtained which was satisfactory for light and electron microscope studies.

Specimen preparation. To find a suitable fixative for electron microscope studies of this yeast, various concentrations of potassium permanganate and osmium tetroxide were tried separately for periods ranging from 10 min to 16 hr. Although a short fixation in 1.5% potassium permanganate resulted in good preservation of the nucleus, cytoplasmic structures were not well preserved. On the other hand, osmic acid alone was found to be a poor fixative for the nucleus and vacuole, the latter being completely destroyed. As a compromise, the suitability of preliminary fixation in potassium permanganate followed by fixation in osmium tetroxide was investigated. After experimenting with various concentrations of the fixatives and varying exposure periods, the following procedure was found to give very good preservation of the cell components. Cells were fixed in a 1.5% aqueous solution of potassium permanganate for 10 min at room temperature, briefly rinsed in distilled water, and fixed in an aqueous solution of 2% osmium tetroxide for 90 min. Specimens were dehydrated by serial passage through a graded alcohol series. Before each change of solution, the material was centrifuged at low speed for 2 min, and, between changes, the centrifuge tube was agitated by hand to disperse the cells.

Partially polymerized butyl methacrylate containing 1.0% benzoyl peroxide as initiator was used for embedding. The specimens were placed in gelatin capsules and polymerized at 55°C for 24 to 48 hr. Ultrathin sections were obtained by means of an LKB 4800 ultratome fitted with a glass knife. Difficulty in sectioning due to the softness of the blocks was partially overcome by leaving them at room temperature for 3 days before sectioning. The technique of Satir and Peachey (1958) was occasionally employed to expand sections. Sections were picked up on 150-mesh copper grids coated with a carbon film, and examined with an Akashi TRS-50 electron microscope.

Cytochemical techniques and light and phase-contrast microscopy. Various cytochemical

methods were used to correlate structures observed in electron micrographs with those observed by light and phase-contrast microscopes. Pinacyanol and dilute Lugol's solution were used for the identification of mitochondria and glycogen, respectively. Lipoidal inclusions were stained with Sudan black B. Observations of the nucleus and mitochondria of living cells were made with a Spencer phase-contrast microscope. HCl-Giemsa and Feulgen techniques (Thyagarajan, 1961) were employed to stain the nucleus after fixation in acetic acid-alcohol (1:3), osmic acid vapor, or iodine-formaldehyde-acetic acid. Iron alum hematoxylin was used to stain the nucleolus (Thyagarajan and Naylor, 1961).

RESULTS

Phase-contrast microscopy of living cells. Observations of living cells with the phase-contrast microscope showed that the nucleus is composed of an optically dense nucleolus surrounded by a shell of uniform material of lower density. The entire structure appeared to be enclosed within a nuclear membrane. Vacuoles were generally absent from actively dividing cells; however, in those cells containing a vacuole, the nucleus was clearly separated from it. Evidence for the presence of a membrane surrounding the vacuole was obtained from observations with dark-field illumination. Granular, rod-shaped, and occasionally long, filamentous structures stainable with pinacyanol were present in the cytoplasm. Small refractile inclusions, stainable with Sudan black B, were also observed. These increased in size during the aging process.

Electron microscopy. The ultrastructure of resting cells and cells undergoing budding with concomitant nuclear division are illustrated in Fig. 1-13. For comparison, phase-contrast micrographs of living as well as fixed and stained cells are also presented. The structure referred to as the nucleus in the electron micrographs was identified on the basis of its behavior during cell division and its cytochemical reactions. Structures such as mitochondria, lipoidal inclusions, vacuoles, internal membranes, the cytoplasmic membrane, and cell wall were also observed.

Nucleus. Electron micrographs reveal that the nucleus of *R. glutinis* is similar to that of *S. cerevisiae* (Hashimoto et al., 1959; Vitols, North, and Linnane, 1961) and *Schizosaccharomyces octosporus* (Conti and Naylor, 1959) in general

appearance and behavior during division (Fig. 1-13 and 15). The nucleus is spherical to ovoid (Fig. 1-13 and 15) and is surrounded by a double membrane. Some of the nuclei contain a dense, more or less spherical structure (Fig. 1-4). Identical structures are visible in the nuclei of living cells (Fig. 1A), and are identified as nucleoli on the basis of their Feulgen-negative staining reaction and their strong affinity for iron alum hematoxylin (Fig. 1B). In some cells the nucleolus shows areas of lesser density, simulating vacuoles, and also some bodies of irregular configuration (Fig. 1-4). The presence of a membrane around the nucleolus is evident in the electron micrographs (Fig. 2-4). Variations in size, shape, and in orientation within the nucleus (Fig. 1-4) are characteristics of the nucleolus which were also observed in living cells. In some cells the nucleolus appears to be double (Fig. 4). Serial sections through the entire nucleolus (Conti, Thyagarajan, and Naylor, *unpublished*) clearly show that in some cells it is not an artifact produced by infolding of the nuclear membrane or intrusion of the cytoplasm into the nucleus, whereas in others, this may be the case.

The nucleoplasm was either granular and homogeneous in appearance (Fig. 1 and 2) or exhibited areas of low electron density (Fig. 4, 5, 7, and 8). These areas were randomly distributed within the nucleus and did not assume any definite ordered arrangement. The appearance of these structures, which seem to contain electron-dense fibrils, is shown in Fig. 5. A similar type of differentiation of the nucleus in sporulating cells of *S. cerevisiae* has been described (Hashimoto et al., 1960). Similar structures were also observed in vegetative cells by Yotsuyanagi (1960) and Vitols et al. (1961). It has been suggested that these areas correspond to the chromosomes of yeast, although definitive studies are still lacking.

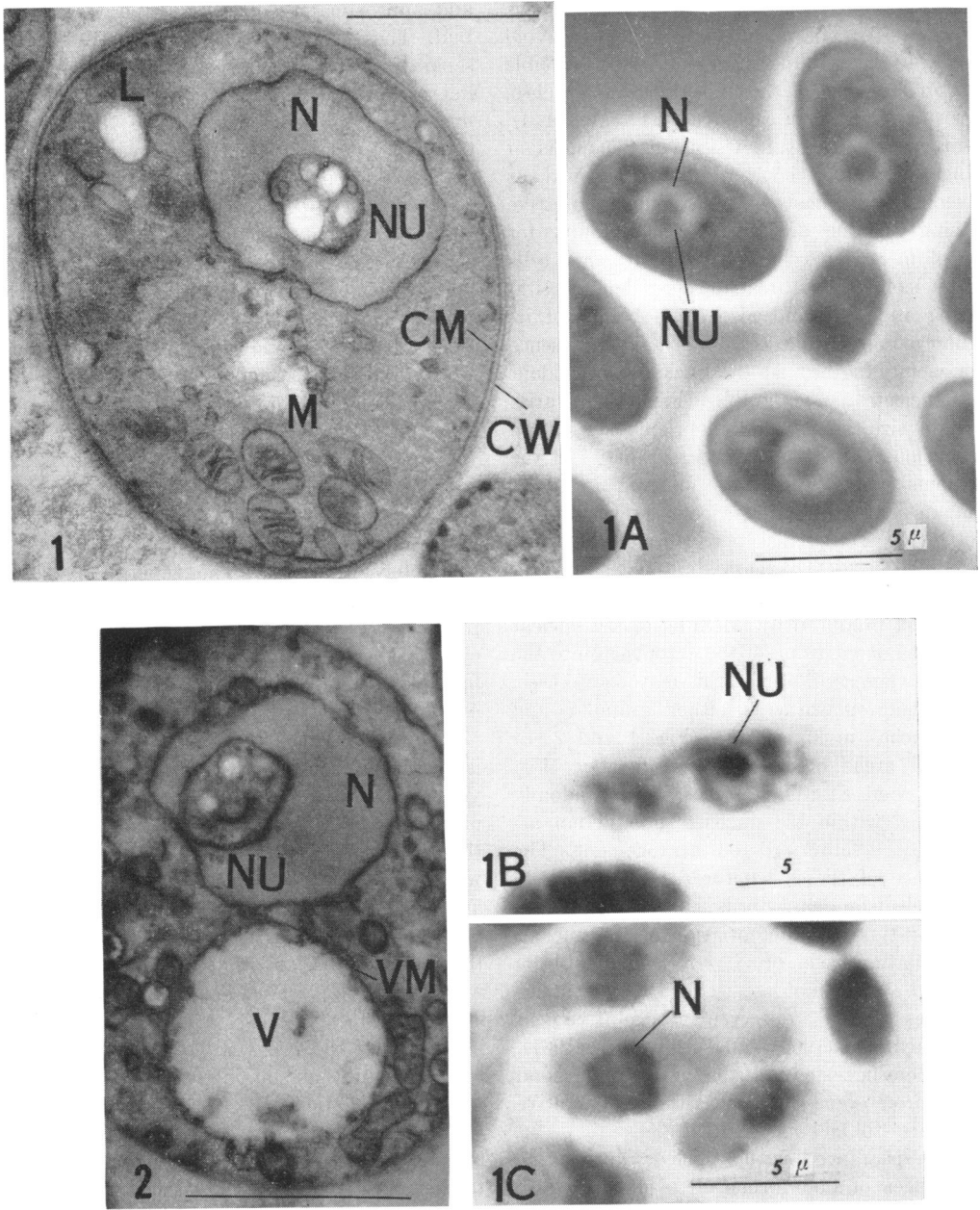
The appearance of the nucleus during the early stages of bud formation is illustrated in Fig. 6-9. A thin membrane, which may be attached to the nuclear membrane, is located in close proximity to the nucleus and was regularly observed (Fig. 6-9). Observations of the dividing nucleus (Fig. 10-13) revealed that, in contrast to the situation found in higher organisms, the nuclear membrane did not disappear during any of the observed stages of cell division. The nucleus, during the process of division, elongates

and approximately half of it migrates into the bud. Then, by a process of constriction and separation, two nuclei are formed. Electron micrographs of serial sections of one dividing nucleus are presented in Fig. 10-13. Although the nucleus appears elongated with a narrow middle region in Fig. 11, the nuclei in the mother cell and bud appear to have separated in Fig. 10A. It is evident that serial sections yield different pictures, illustrating the necessity for exercising caution when interpreting electron micrographs of ultrathin sections. Nuclear division seen in two more serial sections of the same cell are presented at a higher magnification in Fig. 12 and 13. A slight variation in the size and shape of the nucleus is noticeable, and a developing constriction is evident in the middle portion. The result of a single nuclear division is shown in Fig. 15; mother cell and bud each possesses a nucleus, although the cells have not completely separated. The separation of the mature daughter cell from the mother cell appears to be effected by the development of the cytoplasmic membranes, as seen in Fig. 16, followed by the centripetal disintegration of the central portion of the adjoining cell walls.

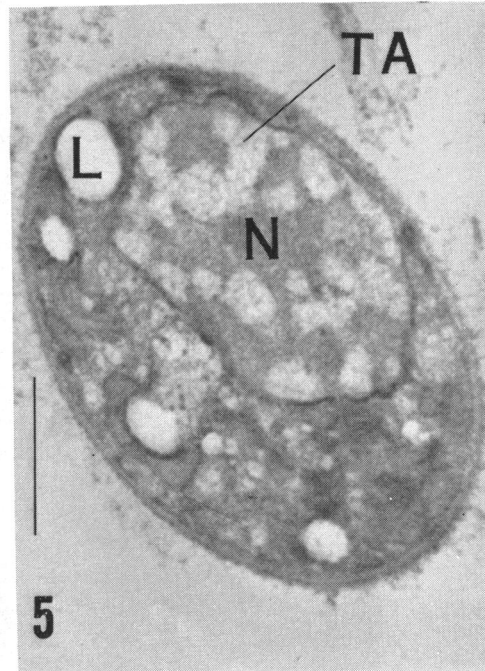
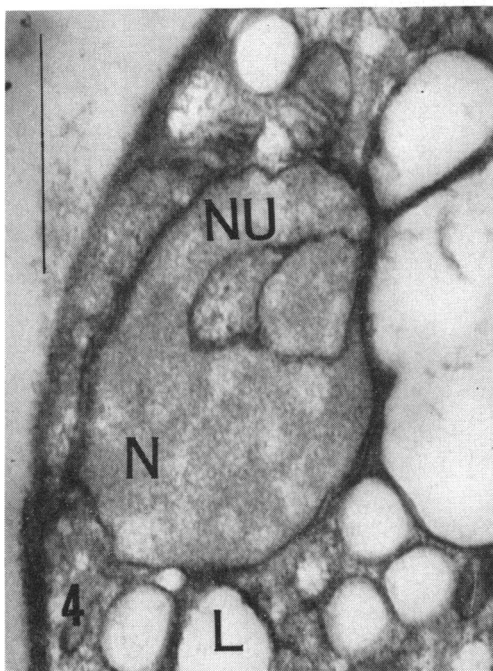
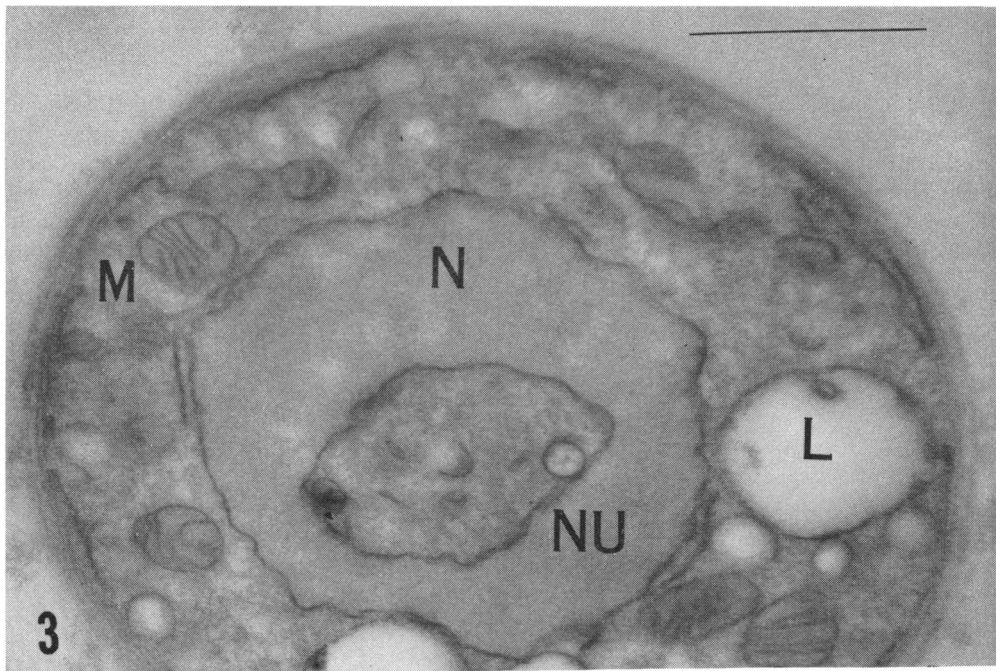
Cell wall. The cell wall of *R. glutinis* (Fig. 1) is much thinner than the cell walls of other yeasts that we have studied with the electron microscope. There is no clear indication that the wall consists of two separate membranes, as was described for *S. cerevisiae* by Bartholomew and Levin (1955). However, a region of electron-opaque material appears to be localized below the outer surface and in the region contiguous with the cytoplasmic membrane. Hashimoto et al. (1959) and others experienced difficulty in observing the cell wall, but the wall of *R. glutinis* requires no staining or special procedures for its visualization.

Cytoplasmic membrane. The cytoplasmic membrane appears to be smooth and closely adherent to the cell wall (Fig. 1). The appearance of this membrane just prior to bud separation is shown in Fig. 16.

Mitochondria. Organelles having typical mitochondrial structure can be seen in Fig. 1, 2, 6, 7, 13, 14, and 16. The number of mitochondria per cell varies over a range of approximately 4 to 20. In thin sections, their profiles appear circular, elliptical, or rod-shaped. The mitochondria measure from 0.4 to 1.0 μ in length and



FIGS. 1-2.



FIGS. 3-5.

FIG. 1. Electron micrograph of a resting cell prior to bud formation, showing the nucleus, nucleolus, mitochondria, cell wall, and cytoplasmic membrane. (A) Phase-contrast photomicrograph of the nucleus in the living cell. Note the optically dense nucleolus surrounded by a shell of uniform material of lower density. (B) Nucleolus stained with iron alum hematoxylin. The chromatin region is not stained. (C) Nucleus stained with HCl-Giemsa. CW = cell wall; CM = cytoplasmic membrane; L = lipoidal inclusion; M = mitochondria; N = nucleus; NU = nucleolus.

FIG. 2-4. These electron micrographs illustrate the variation in the size and shape of the nucleolus and also its orientation within the nucleus. The nucleolus appears double in Fig. 4. The electron-transparent vacuole possesses a membrane in Fig. 2. VM = vacuolar membrane; TA = transparent areas within the nucleus.

FIG. 5. Areas of lower electron density containing dense fibrils are seen within the nucleus.

0.2 to 0.4 μ in diameter. *Cristae mitochondriales* are prominent and extend in various directions within the mitochondrion (Fig. 6, 13, 14, and 15). A similar observation was made by Vitols et al. (1961), while studying *S. cerevisiae*. Agar and Douglas (1957) reported that the mitochondrial

cristae of *S. cerevisiae* run parallel to the long axis of the mitochondrion. As suggested by Palade (1953), the cristae appear to be formed by the infolding of the inner membrane of the mitochondrion (Fig. 6, 13, and 14). Electron micrographs of dividing cells show the migration

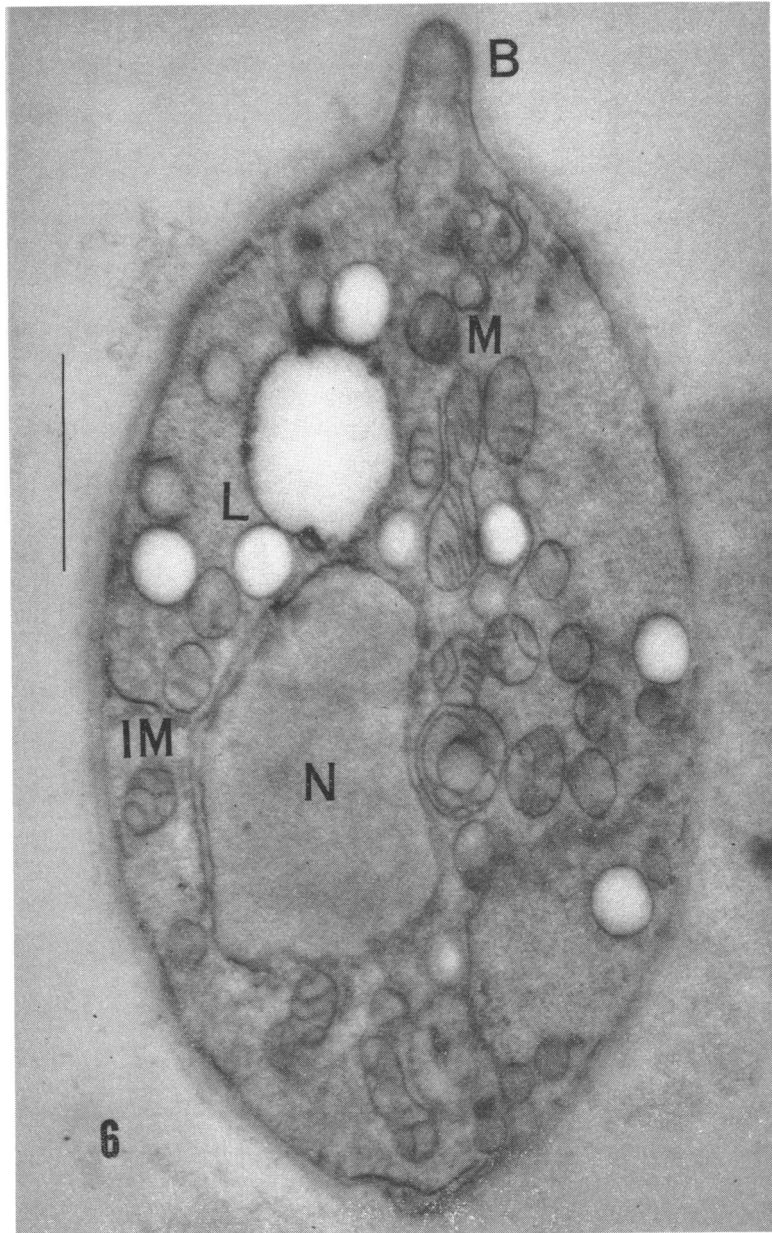


FIG. 6. Initiation of bud formation. Note the shape of the nucleus, the distribution and structure of mitochondria, and the presence of internal membranes. B = bud; IM = internal membrane.

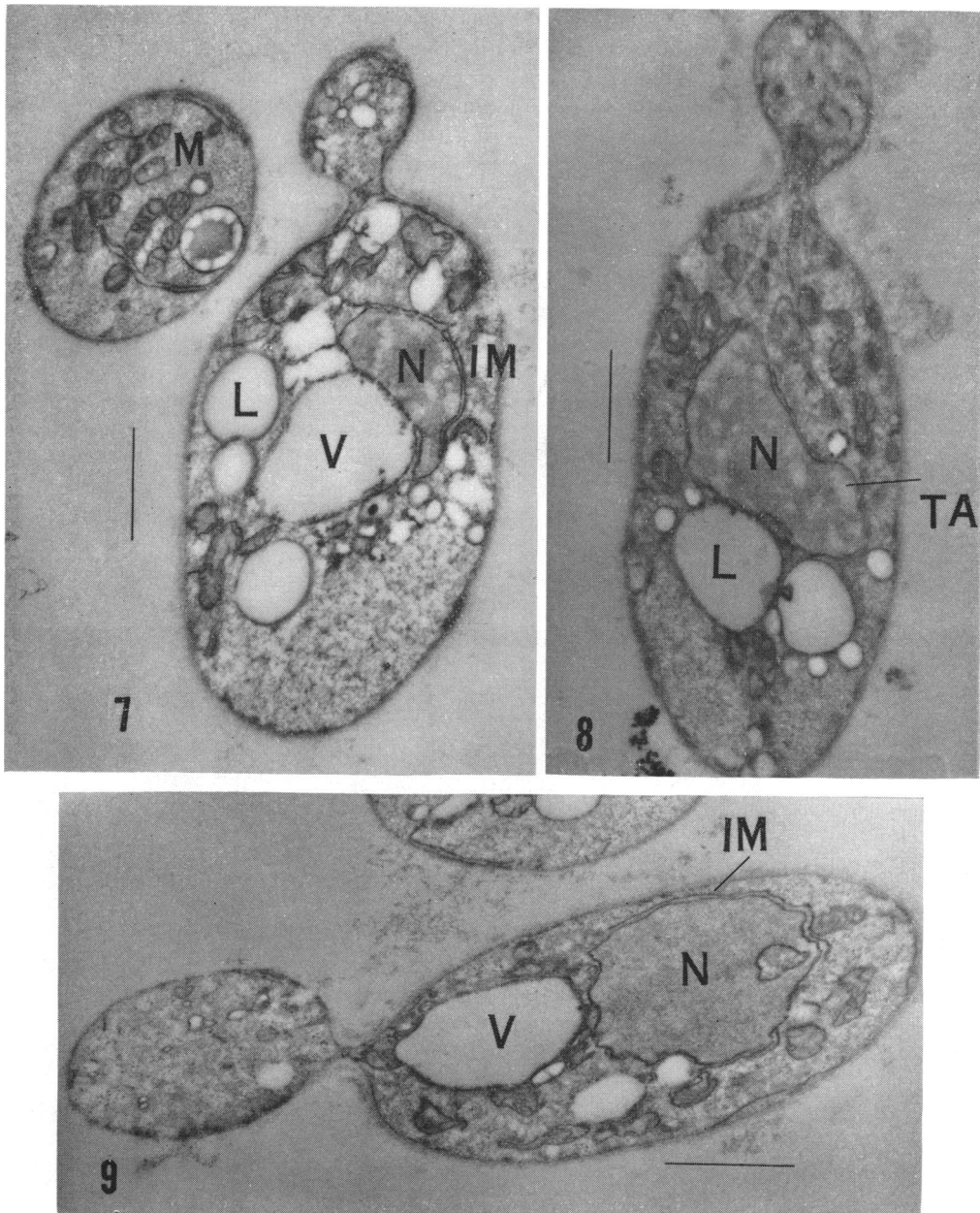


FIG. 7-9. The appearance of the nucleus during the early stages of bud formation. A thin membrane, attached to the nuclear membrane and located in close proximity to the nucleus, is clearly visible in all these electron micrographs. Areas of low electron density within the nucleus are visible in Fig. 7 and 8. The mitochondria appear to migrate into the bud prior to the onset of nuclear division. Note the intranuclear inclusion in Fig. 9.

of mitochondria into the bud even before the onset of nuclear division (Fig. 7-9). The presence of some mitochondria in very young buds points to the fact that developing buds initially receive them from the mother cell.

At certain stages mitochondria seem to aggregate near the nucleus (Fig. 6), and there is some evidence for the presence of connections of mitochondria with the nuclear membrane as well as with other mitochondria (Fig. 6 and 7).

Often the internal membranes established contact with the mitochondria and cytoplasmic membranes (Fig. 6 and 9).

Lipoidal inclusions. In addition to mito-

chondria, the cytoplasm contains some well-defined electron-transparent areas. These have been identified as lipids on the basis of their empty appearance in electron micrographs, their

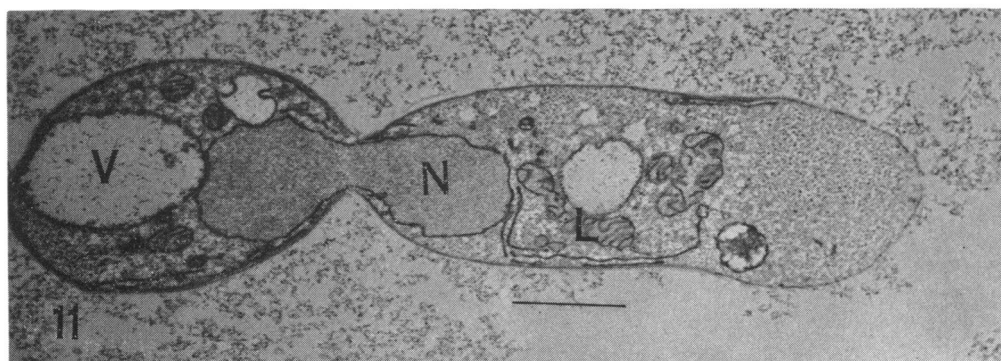
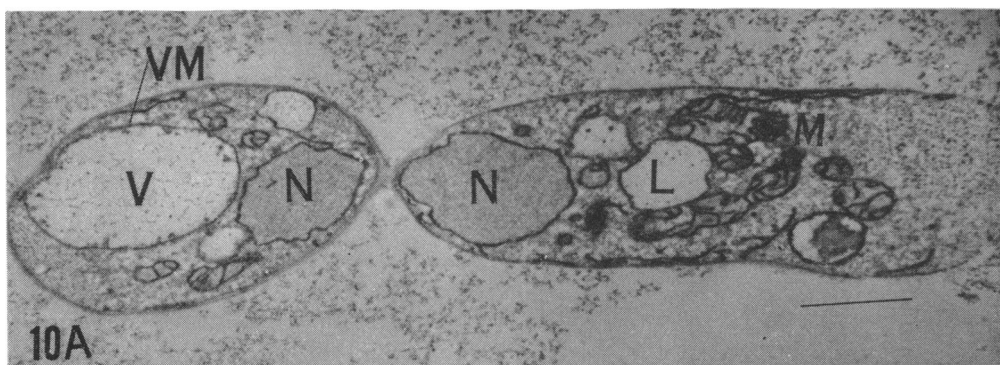
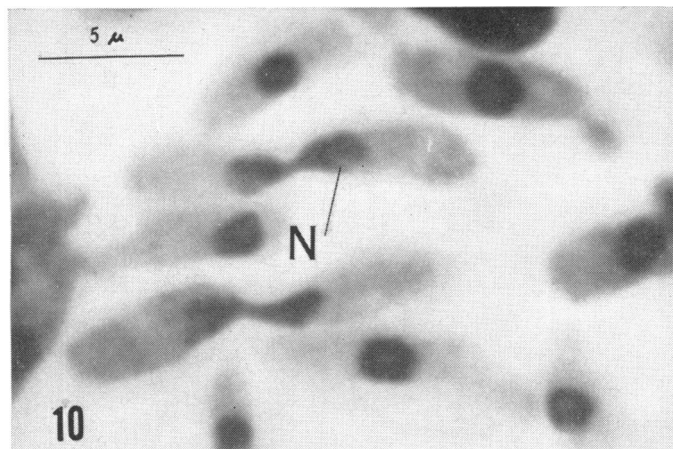


FIG. 10 and 11. Fig. 10 is a light micrograph of the dividing nucleus stained with HCl-Giemsa. Fig. 10A and 11 are serial sections of a dividing cell. In Fig. 10A the nucleus has migrated into the bud, with the nuclear membrane still intact. In Fig. 10A the nuclei in mother cell and bud appear to have separated due to the plane of sectioning. The vacuolar membrane is clear in the bud in Fig. 11.

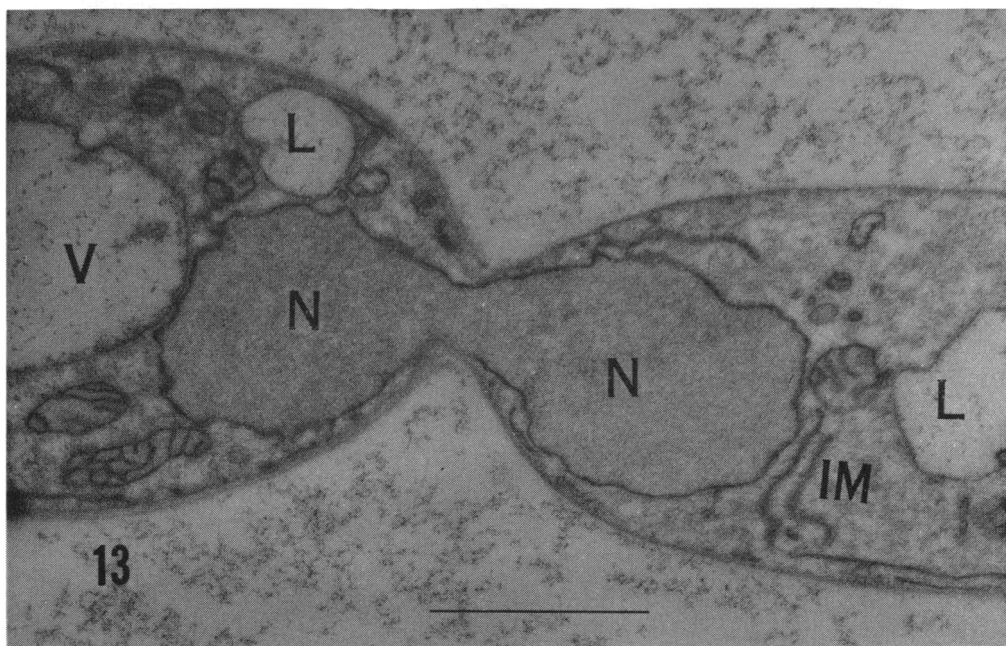
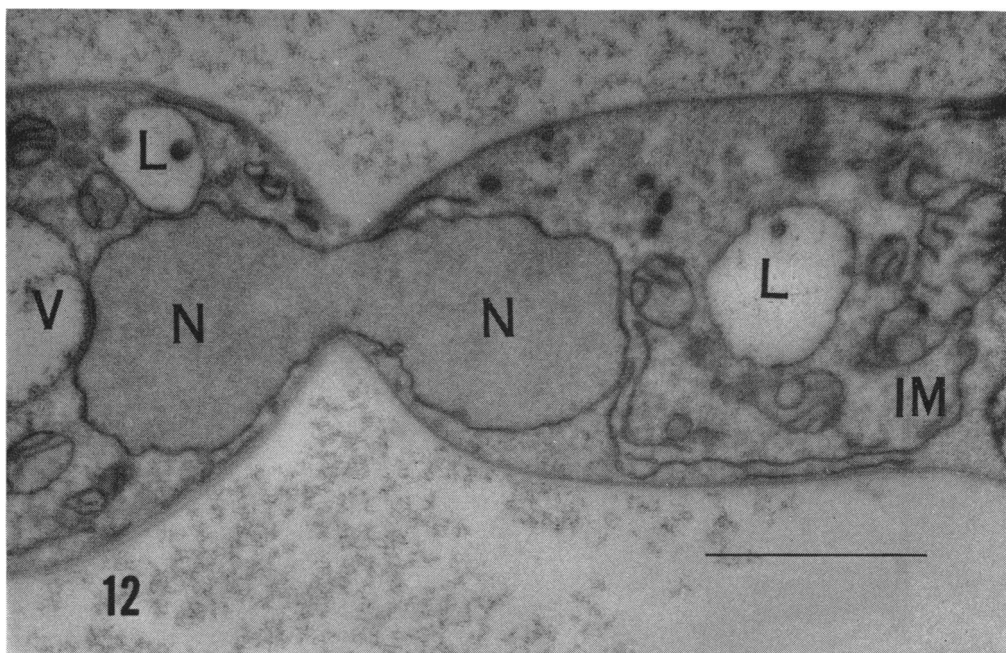


FIG. 12 and 13. Parallel sections of the cell shown in Fig. 10A and 11, illustrating the shape of the dividing nucleus. A slight variation in the shape and size of the nucleus is noticeable. Note the developing constriction in the middle part of the nucleus, the structure of the mitochondria, and the presence of internal membranes. Examination of Fig. 10A, 11, 12, and 13 show that the nucleus and vacuole have separate membranes.

presence in living cells, and their stainability with Sudan black B (Fig. 1, 5, and 6-10). Their size and number vary with age and cultural conditions.

Vacuole. The general absence of vacuoles in actively dividing cells was noted from the light and phase-contrast microscope studies. When present, the vacuole in electron micrographs is

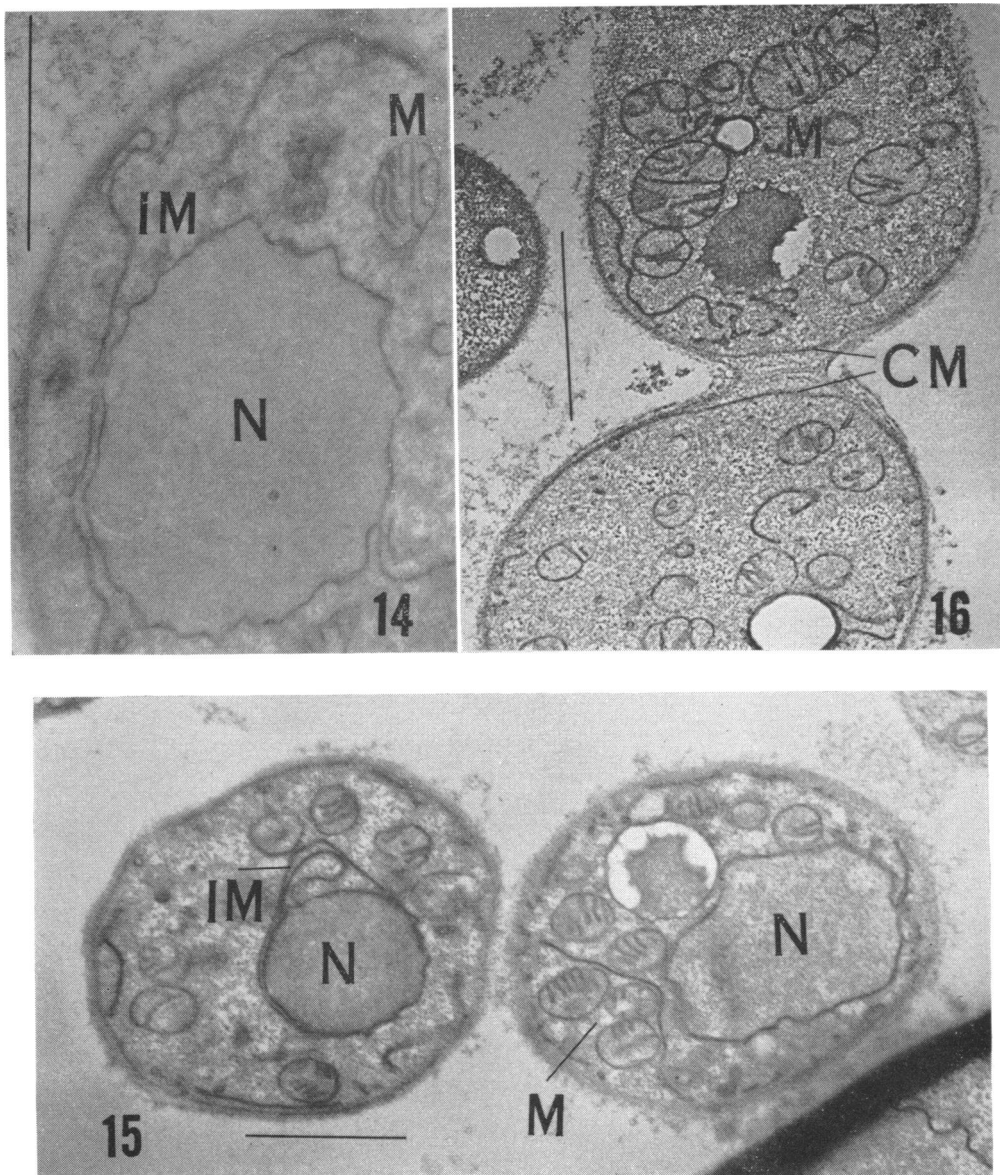


FIG. 14. This electron micrograph shows the presence of internal membranes and their connections with the nuclear and cytoplasmic membranes. Note the discontinuities ("nuclear pores") in the nuclear membrane.

FIG. 15. Mother cell and bud each possesses a nucleus, but the cells have not completely separated. The internal membrane in the bud is attached to the nuclear membrane.

FIG. 16. Cells in late stages of division. The cytoplasmic membranes have been formed prior to cell separation.

seen as an electron-transparent area within the cell, and appears to be separated from other cell constituents by a membrane (Fig. 2 and 11). The shape and size of the vacuole varies with the age and physiological state of the cell. Occasionally, the vacuole contains granular material, which may be volutin. The structure of the vacuole is similar to that of *S. cerevisiae* (Hashimoto et al., 1959; Vitols et al., 1961).

Internal membranes. Evidence for the presence of an extensive internal membrane system is shown in Fig. 6-16. A membrane was regularly observed (Fig. 6-9) along the periphery of the nuclear membrane. This membrane appears to be connected to the nuclear and cytoplasmic membrane as well as to the mitochondria (Conti, Thyagarajan, and Naylor, *in press*).

DISCUSSION

The nucleus of *R. glutinis* has a well-defined limiting membrane, and contains intranuclear structures in the form of a nucleolus and electron-transparent areas. The nucleolus has been reported in electron micrographs of *S. cerevisiae* by Yotsuyanagi (1959) and of the yeast phase of *Histoplasma* by Edwards, Hagen, and Edwards (1959). The studies of Guilliermond (1920), Kater (1927), Lietz (1951), Necas (1960), and Robinow (1961) established, with the light microscope, the presence of a nucleolus in yeast. Although the nucleolus in higher plants has been examined in great detail with the electron microscope (Lafontaine, 1958), very little is known about the structure and behavior of the yeast nucleolus. Light-microscope studies of iron alum hematoxylin-stained cells of *R. glutinis* showed the division of the nucleolus by elongation and constriction during nuclear division (Thyagarajan and Naylor, 1961). The behavior of the nucleolus could not be followed in living cells under the phase-contrast microscope because of various technical difficulties. Similarly, in the present study the behavior of the nucleolus during nuclear division could not be observed in ultrathin sections. This may have been due to the plane of sectioning and the thinness of the sections or to the absence of the nucleolus during some stages of cell division.

With regard to the presence of chromosomes in yeast, it can only be surmised that the electron-transparent intranuclear areas may correspond to chromosomes stainable with the

Feulgen technique. Our results agree with those obtained with sporulating cells of *S. cerevisiae* by Hashimoto et al. (1960) and with vegetative cells of the same species by Yotsuyanagi (1960). Such masses of electron-transparent material have also been reported in various bacteria, algae, and in a deuteromycete by McAlear and Edwards (1959). Further refinement in techniques may enable one to observe the exact nature of chromosomes in the yeast nucleus.

During nuclear division the nuclear membrane was found to persist, and the nucleus appears to divide by a simple process of elongation and constriction similar to that observed in living and stained cells (Thyagarajan and Naylor, 1961). The persistence of the nuclear membrane during the complete division process in *R. glutinis* is in conformity with earlier reports made by Hashimoto et al. (1959) on *S. cerevisiae* and Conti and Naylor (1959) on *S. octosporus*. Similar electron-microscope observations have been made on the yeast phase of *H. capsulatum* (Edwards et al., 1959) and on *Neurospora crassa* (Shatkin and Tatum, 1959).

Although occasional openings in the nuclear membranes were observed, these are not considered to be "nuclear pores" since they may have been produced during specimen preparation. Nuclei which give the appearance of being well preserved lack these openings. Vitols et al. (1961) reported that the nucleus of *S. cerevisiae*, like the nuclear envelope of an interphase cell of other tissues, is enclosed within a pair of unit membranes. Our studies to date indicate that the nuclear envelope of *R. glutinis* is a unit membrane. Further studies on the fine structure of the nuclear envelope of *R. glutinis* are in progress.

The present study is further evidence that the vacuole is not a permanent cell inclusion. The absence of vacuoles in actively dividing cells of *R. glutinis*, the Feulgen-negative nature of the vacuoles when present in older cells, their affinity for neutral red in living cells (Thyagarajan, 1958), and the possession of separate membranes by the vacuoles and nuclei lend further support to the generally accepted view that the vacuole is not an integral part of the nuclear apparatus of yeast, as had been proposed by Lindgren, Williams, and McClary (1956). Previous studies by Hashimoto et al. (1958, 1959), Conti and

Naylor (1959, 1960*a,b*), and Vitols et al. (1961) have also led to a similar conclusion.

Mitochondria with typical cristae occur in the cytoplasm of *R. glutinis*, and their structural details are identical to those reported for higher organisms (Palade, 1953; Sjostrand, 1953; Novikoff, 1961). Migration of mitochondria into the bud, even before the passing of the nucleus, was evident in many instances. These observations point to the facts that mitochondria in developing buds arise from pre-existing ones, and are produced only in the cells which possess a nucleus. The polymorphic nature of mitochondria is shown in the electron micrographs, and the occurrence of rod or thread-like forms in yeast (Thyagarajan and Subramaniam, 1960) cannot be disputed.

Dempsey (1956) has pointed out the inadequacy of the "fission" theory of mitochondrial multiplication and development in higher organisms. He also contended that the theory of fabrication of new mitochondria from intracellular membranes in tissues of higher animals is not adequately substantiated. Detailed studies of the origin and multiplication of mitochondria in yeast may throw more light on this problem.

Agar and Douglas (1957) reported that portions of the cytoplasmic membrane of *S. cerevisiae* were invaginated. In cells fixed with potassium permanganate and treated with uranyl nitrate, the cytoplasmic membrane appears as two dense lines separated by a dense layer (Vitols et al., 1961). The cytoplasmic membrane of *R. glutinis* is closely adherent to the cell wall of both resting and actively dividing cells. These divergent observations may be due to inherent differences in the yeasts studied, differences in fixation methods employed, or differences in the physiological age of the cells prepared for sectioning.

The presence of internal membranes in the cytoplasm and the connection of the nuclear membrane with them is not surprising in view of similar observations on budding yeast-phase cells of *Histoplasma* (Edwards et al., 1959) and sporulating cells of *S. cerevisiae* (Hashimoto et al., 1960) and *S. octosporus* (Conti and Naylor, 1960*b*). Recent studies on *Streptomyces coelicolor* (Glauert and Hopwood, 1960), *Bacillus subtilis* (Tokuyasu and Yamada, 1959), and *Mycobacterium leprae* (Brieger, Glauert, and Allen, 1959) show that the cytoplasm of bacteria also contains complex membrane systems. The con-

tinuity of mitochondria with the cytoplasmic and nuclear membranes was also seen in this yeast. The significance of these membranes, often referred to as endoplasmic reticulum, and their role in cell metabolism have been discussed in detail by Porter (1961).

A centriole was not observed in any of our electron micrographs. Failure to observe this structure does not disprove its existence. The inability to observe such structures may be ascribed to a variety of factors (Moses, 1956; Cosslett, 1958; Conti and Naylor, 1959).

Our observations of *R. glutinis* are in general agreement with published descriptions of other yeasts. However, improved fixation and the suitability of this organism for electron microscope investigations have yielded additional information on yeast ultrastructure. Detailed descriptions of the mitochondrial ultrastructure and intranuclear structures will be presented elsewhere.

Criticisms of the use of osmic acid and potassium permanganate fixation for electron microscope studies of yeast ultrastructure have been presented recently (Mundkur, 1960*b*). It was stated that "none of these workers were [*sic*] successful in preserving the vacuole, the total collapse of which may be considered as an indicator of the limitation in preservation of fine structure accruing from such fixatives." Reference to the micrographs presented by Agar and Douglas (1957), Yotsuyanagi (1960), and others, as well as those presented in this paper, does not support the claim of "total collapse" of the vacuole. The assumption that all yeast cells must have vacuoles is subject to criticism. Potassium permanganate as a fixative has also been questioned on the basis that "both the nucleus and the cytoplasm stain with strikingly equal intensity such that, were it not for the presence of a clearly stained nuclear boundary, the nucleus would be indistinguishable owing to its granular appearance being identical in texture with that of the cytoplasm."

It seems obvious that preservation of membranous systems and ultrastructure is more important than the detection of textural differences whose existence is doubtful. Although the use of freeze drying and staining techniques for electron microscopy may prove to be valuable tools in the study of yeast ultrastructure, the advocacy of these techniques to the exclusion

of others is questionable. Reference to the micrographs presented to date utilizing the freeze-drying technique (Mundkur, 1960*a,b*) makes this clear.

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