CYTOLOGY OF *RHODOTORULA GLUTINIS*

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ABSTRACT

THYAGARAJAN, T. R. (Cornell University, Ithaca, N. Y.) AND H. B. NAYLOR. Cytology of *Rhodotorula glutinis*. J. Bacteriol. **83**:127–136. 1962.—The structure and manner of division of nuclei in actively dividing cells of *Rhodotorula glutinis* were studied with the phase contrast microscope. The nucleus consists of a dense central body, surrounded by a shell of optically uniform material of low density. The entire structure is enclosed within a nuclear membrane. Various fixation and staining techniques were employed to confirm the observations made from living cells. Since the dense central body is Feulgen-negative and is readily stained by iron alum hematoxylin, it is identified as the nucleolus. The material surrounding the nucleolus has no marked affinity for hematoxylin but is Feulgen-positive and stains intensely with Giemsa and basic fuchsin. The nucleus appears to divide by a process of elongation and constriction during which roughly half of the nucleolus, along with the surrounding chromatin, passes into the bud. The nuclear membrane was found to persist during all stages of division. Vacuoles were seldom observed in actively dividing cells. The nucleus of *R. glutinis* is similar in structure to the nuclei of higher organisms, but its behavior during division is quite different.

Although there is general agreement as to the proper identification of the nucleus in yeast, there appears to be no unaniminity of opinion about the exact structure of the nucleus. According to Mundkur (1954, 1960) and Townsend (1961), the nucleus is a homogeneous vesicle without any structural details. On the other hand, Royan (1956, 1958*a*,*b*), Thyagarajan (1959, 1961), and Subramaniam (1960), from observations made on living as well as fixed and stained cells, have shown the nucleus to be composed of chromocenters, nucleolar equivalents, and a nuclear membrane. Necas (1960) reported having seen a nucleolus inside the nucleus of living and stained yeast protoplasts. In the early studies of the structure of yeasts with the electron microscope, no indications of structural differentiation of the nucleus during any of the stages of division were found (Agar and Douglas, 1957; Hashimoto, Conti, and Naylor, 1958, 1959; Conti and Naylor, 1959, 1960*a*,*b*). More recent investigations (Hashimoto et al., 1960; Vitols, North, and Linnane, 1961), however, have indicated that some differentiation can be observed by this method, and Yotsuyanagi (1959, 1960) has presented electron micrographs showing structures which were interpreted to be chromosomes and nucleoli in the nuclei of *Saccharomyces cerevisiae*.

Reviewing the present status of our knowledge of the structure of the yeast nucleus, Subramaniam (1960) pointed out the necessity of the study of nuclei in living cells to correlate observations made on fixed and stained cells. Since Subramaniam et al. (1959) have shown that it is possible to observe the nucleus in living cells under certain cultural conditions, an attempt was made to study the nuclei in living *R. glutinis* and to compare the structure with fixed and stained preparations. This paper embodies these observations.

MATERIALS AND METHODS

R. glutinis, obtained from L. J. Wickerham, was grown in different types of media to determine optimal conditions for observing the nuclei in living cells. When cells were cultured in rich media containing glucose, peptone, and yeast extract, or barley malt wort (sp gr 1.02), the cells had a tendency to accumulate large inclusion bodies stainable with Sudan black B. In cells filled with these fat bodies, the nucleus could not be located, and it was found necessary to culture cells bereft of these bodies. Different concentrations of barley malt wort were tried, and finally it was found that cells could be grown without any visible fat bodies when a dilute

medium of barley malt wort (sp gr 1.005), adjusted to pH 4.6, was used as the culture medium. Cells were kept actively growing at room temperature in this liquid medium by subculturing every 2 days. Examination of the living cells under the phase contrast microscope showed that the nucleus could be seen in a majority of cells in a 24 to 48 hr culture. To observe and photograph the nuclei in living cells, a loop of material was removed from the top layer of the culture tube, placed on a clean slide, and mounted under a square cover glass. Excess medium was removed from the sides of the cover glass by means of filter paper strips before sealing with paraffin. Observations of living cells were made with a Spencer phase contrast microscope, and photographs were taken with a Leica camera attachment.

To obtain enough material for smearing slides, a 48-hr test tube culture was added to a Roux flask containing 150 ml of medium, and the cells were allowed to grow undisturbed for 48 hr.

Fixation and staining techniques. Cells from 48-hr cultures were sedimented by centrifugation, washed in distilled water, and smeared in thin films on slides lightly coated with albumin. Considerable difficulty, due to clumping, was encountered in smearing cells uniformly. For good and uniform preservation of structural details and for obtaining consistently uniform staining, it was found necessary to smear the cells gently, without applying too much pressure. Slides were fixed either in acetic acid-alcohol (1:3) for 30 min followed by osmium tetroxide vapors for 2 to 3 min, or in a compound fixative containing iodine, formaldehyde, and acetic acid (Thyagarajan, 1961) for 1 hr. The slides were then stored in 70% alcohol. Prior to staining they were washed with running tap water for 30 min and then rinsed in distilled water. The stains used were HCl-Giemsa, Feulgen, iron alum hematoxylin (Thyagarajan, 1961), basic fuchsin, and Azure A-SO_2 (DeLamater, 1948, 1951).

For comparative purposes, cells were also treated with ribonuclease before staining the nuclei with Giemsa or the Feulgen reagent. Cells fixed in acetic acid-alcohol for 30 min and washed in water were placed in a solution of crystalline ribonuclease (Worthington) containing 1 mg of enzyme per ml of distilled water and incubated for 3 hr at 37 C. After incubation these preparations were rinsed in distilled water and stained with Giemsa or the Feulgen reagent.

Mitochondria were stained with pinacyanol, glycogen with Lugol's iodine, and fat bodies with Sudan black B.

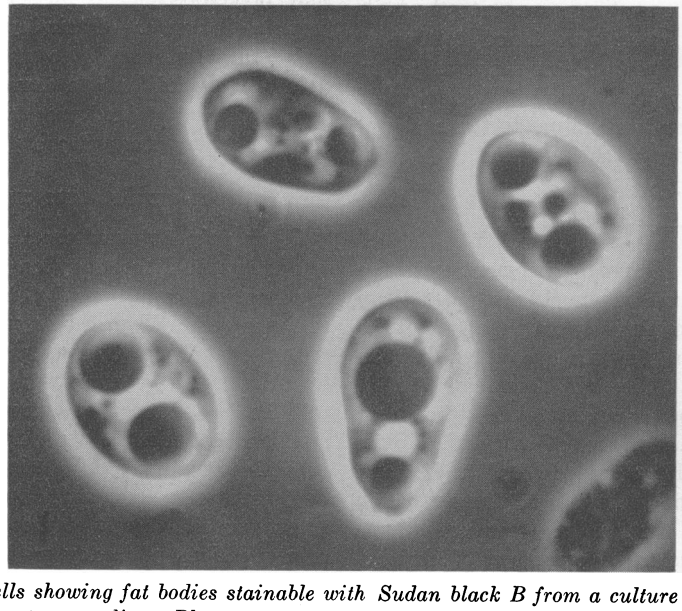

FIG. 1. *Living cells showing fat bodies stainable with Sudan black B from a culture grown on glucose, peptone, and yeast extract medium. Phase contrast.* × 4,500.

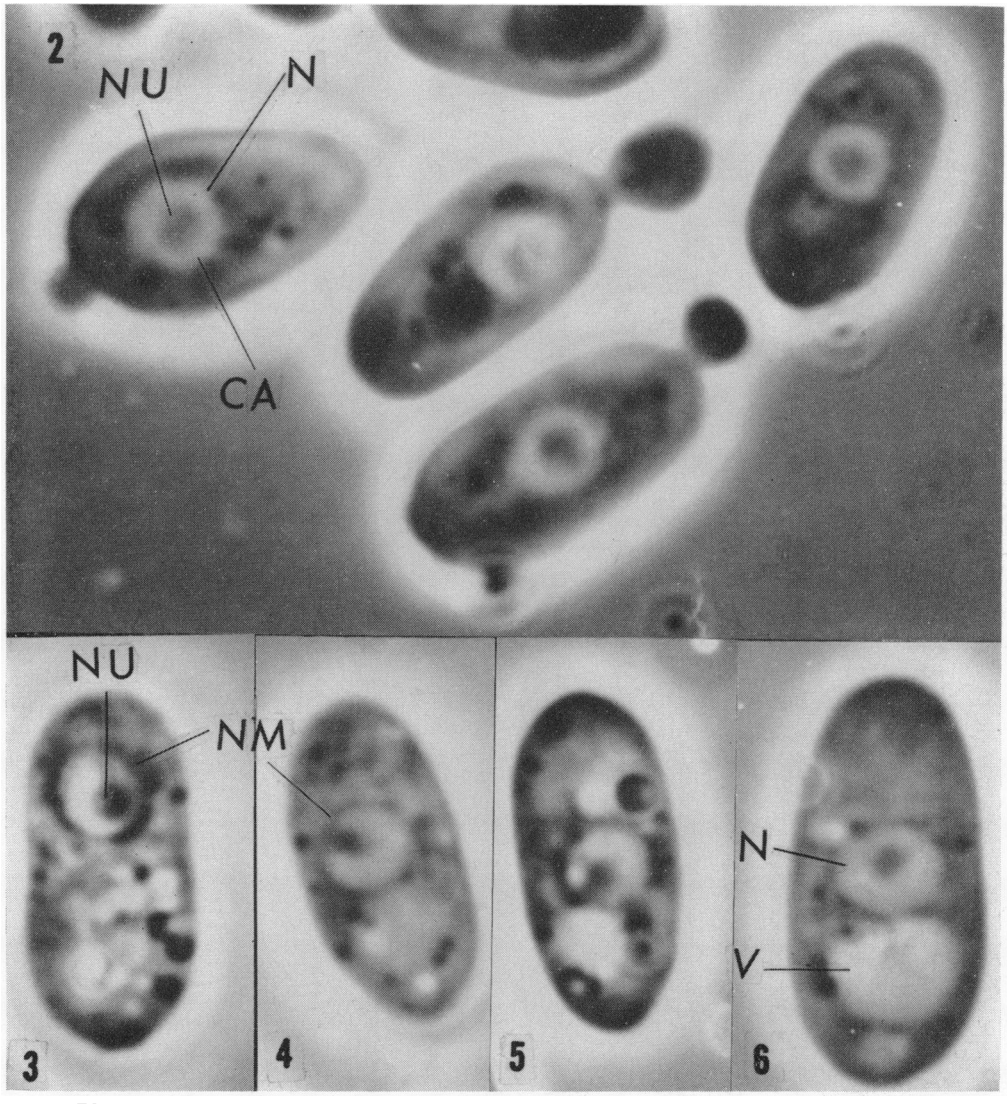

FIG. 2. *Phase contrast micrographs illustrating the nuclei of living cells from a dilute barley malt wort medium.* × *5,500. N = nucleus; NU = nucleolus; CA = chromatin area.*

FIG. 3, 4, 5, and 6. *The nuclear membrane is visible in these living cells. Phase contrast.* × *5,500. NU = nucleolus; NM = nuclear membrane; V = vacuole.*

RESULTS

Observations of living cells with phase contrast microscope. The nuclei could not be located in living cells filled with fat bodies (Fig. 1) produced during growth in rich media or in barley malt wort (sp gr 1.02). When cultured in dilute barley malt wort medium, it was possible to obtain cells without large fat bodies. Examination with the phase contrast microscope revealed that the nucleus could be seen in a majority of cells actively growing in a 24 to 48 hr culture. The living nucleus is composed of an optically dense spherical central body, the nucleolus, surrounded by a shell of optically uniform material of lower density (Fig. 2). Visible structures could not be seen in the clear area surrounding the central body. Vacuoles were generally absent in these young cells. Granular and rod-shaped mitochondria were often found in the cytoplasm (Thyagarajan and Subramaniam,

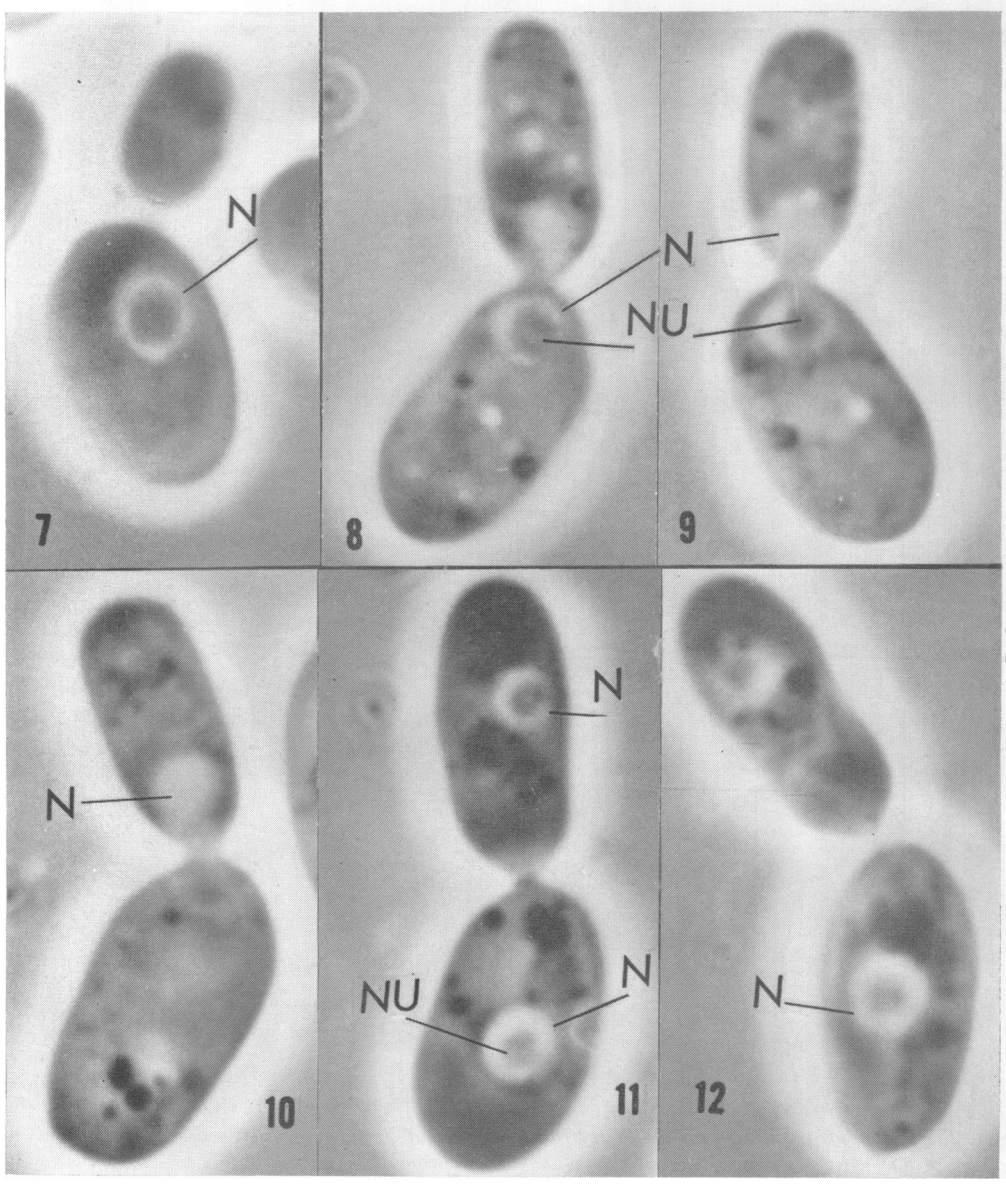

FIG. 7, 8, 9, 10, 11, and 12. *Various stages of nuclear division as observed in living cells. Phase contrast.* × *5,500. N = nucleus; NU = nucleolus.*

1960). The central bodies of nuclei were not optically homogeneous, and appeared to contain small clear areas within their mass. Sometimes the central body presented a duplex appearance. The nuclear membrane was not visible in young cells, but when the cells were allowed to age, i.e., 48 to 96 hr, the membrane could be seen (Fig. 3, 4, and 5). During the aging of cells, small fat bodies were found to accumulate in the cytoplasm, but these did not obscure the nucleus. In cells with vacuoles (Fig. 5 and 6) the nucleus was situated outside the vacuole. The presence of a membrane around the vacuole was evident from examination of living cells under dark-field illumination. This membrane separated the vacuole from the nucleus.

At the onset of nuclear division, the nucleus is often found very near the budding end of the

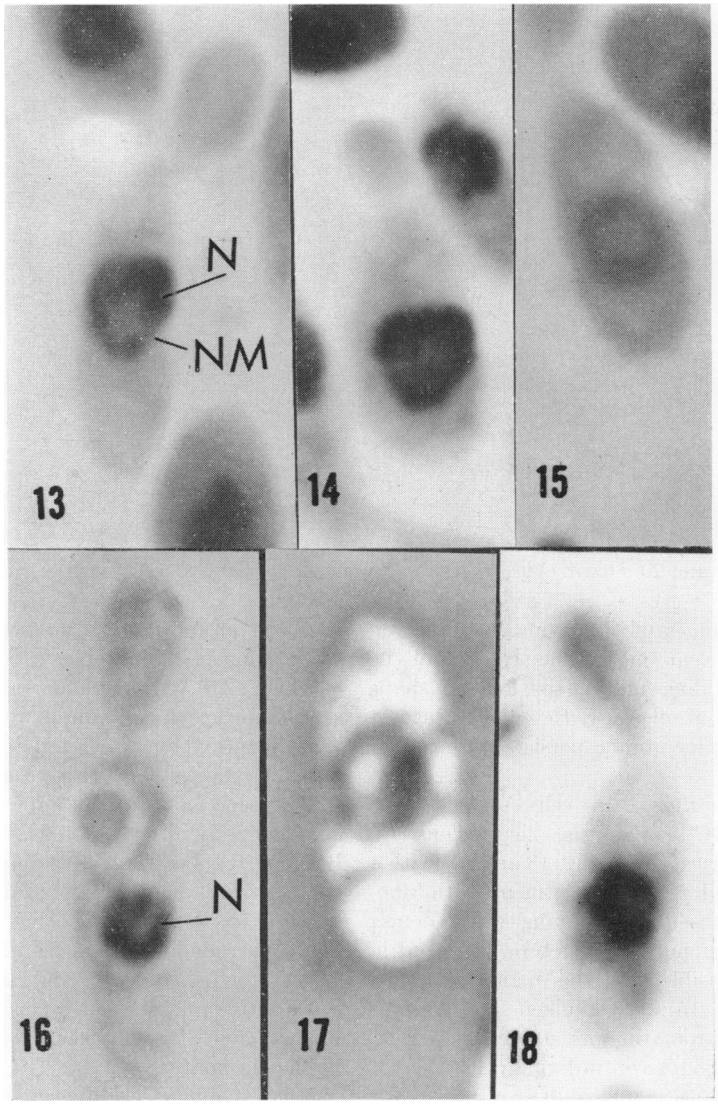

FIG. 13 and 14. *Acetic alcohol-Giemsa.* × *5,500. N = nucleus; NM = nuclear membrane*
FIG. 15. *Acetic alcohol-fixed cell, treated with ribonuclease and stained with Giemsa.* × *5,500*
FIG. 16. *Acetic alcohol-Feulgen.* × *5,500*
FIG. 17. *Acetic alcohol-Feulgen. Phase contrast.* × *5,500*
FIG. 18. *Iodine-formaldehyde-acetic acid and basic fuchsin.* × *5,500*

cell (Fig. 7). Then the optically uniform material which appears as a halo surrounding the dense central body slowly elongates and flows into the bud, taking on a more or less dumbbell appearance (Fig. 8, 9, and 10). Constriction of the nucleus then occurs, and approximately half of it passes into the bud. It was not possible to follow the division of the central body in living cells, but in hematoxylin-stained preparations the elongation and constriction of this body was observed and from this it is presumed that a similar process occurs in living cells also. At the end of nuclear division, each mother cell and bud show a similar nuclear structure, each with its own dense central body surrounded by an optically clear area (Fig. 11 and 12). Spindles, centrosomes, metaphase plates, or individual chromosomes were not observed during

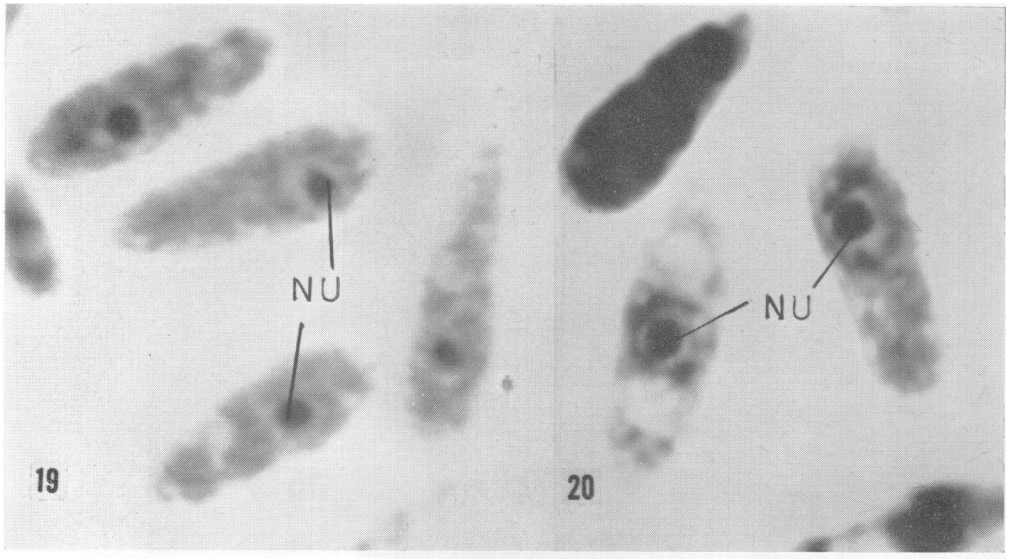

FIG. 19 and 20. *Osmic acid vapor-iron alum hematoxylin.* × *5,500. NU = nucleolus*

the divisional stages of the living nuclei. Even though the nuclear membrane could not be observed in dividing nuclei, the general shape and outline maintained by the nuclei suggest that the nuclear membrane persists during division.

Stained preparations. In cells stained with Giemsa after HCl hydrolysis, the nucleus appeared as a spherical mass (Fig. 13 and 14) with a central unstained region. Treatment with ribonuclease and subsequent staining with Giemsa revealed a similar nuclear structure. The central body was not visible, and this region remained unstained (Fig. 15). In Feulgen preparations (Fig. 16), the chromatin was stained as a ring, but the central body seen in living cells was not stained and this area appeared as a gap in the nucleus. The Feulgen-negative nature of the central body suggested that it might be the nucleolus. Figure 17 is a Feulgen preparation photographed under the phase contrast microscope. Here also the unstained central region is evident. The nuclear membrane and the chromatin lining it are clearly visible. Basic fuchsin staining revealed a similar nuclear picture (Fig. 18). In contrast to this, however, interdivisional nuclei stained with iron alum hematoxylin showed the nucleus to be composed of a densely stained central body surrounded by an unstained area. A group of cells in Fig. 19 illustrates this condition. In a bivacuolate cell, the nucleus with the prominent central body is situated in the portion of the cytoplasm between the vacuoles (Fig. 20). Although it was not possible to see the nuclear membrane in all the cells stained with hematoxylin, due to the difficulty experienced in destaining with iron alum solution, the presence of a membrane was evident in cells properly differentiated (Fig. 21 and 22). Because the body which stained brightly with hematoxylin was Feulgen-negative, it was considered to be the nucleolus. The unstained region surrounding the nucleolus had no marked affinity for hematoxylin, and, since it was Feulgen-positive and stained intensely with Giemsa, this portion of the nucleus was considered to be the chromatin.

Studies of nuclear division in *R. glutinis* by means of various nuclear staining techniques (Giemsa, Feulgen, basic fuchsin, and Azure A) yielded results comparable to those described for living cells observed with the phase contrast microscope. Figures 23, 24, and 25 are photomicrographs of cells stained with Giemsa at different stages of nuclear division. A comparison of these pictures with Fig. 7 through 12 emphasizes the similar appearance of the nuclear apparatus during division, as revealed by the different methods.

As shown earlier, the central body of the nucleus is Feulgen-negative and stains intensely only with hematoxylin. In view of this, hema-

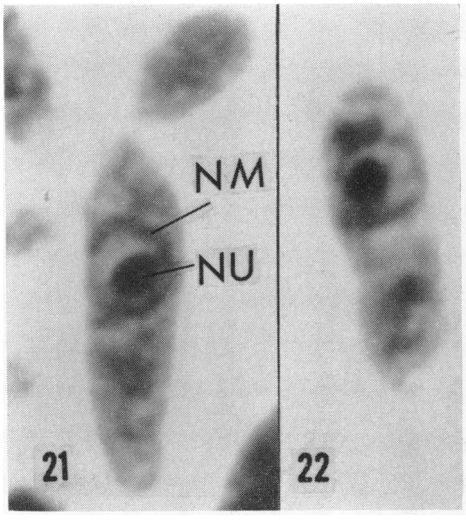

FIG. 21 and 22. *Acetic alcohol-iron alum hematoxylin.* ×*5,500. NM = nuclear membrane; NU = nucleolus.*

toxylin was employed to follow the behavior of the nucleolus during nuclear division. Like the chromatin in the nucleus, the nucleolus appeared to divide by a simple process of elongation (Fig. 26) and constriction (Fig. 27) in hematoxylin-stained cells. The presence of a nucleolus in the mother cell and its bud is illustrated in Fig. 28. The unstained region surrounding the nucleolus is evident.

DISCUSSION

The purpose of this work was to compare observations made on nuclear structure and behavior in living *R. glutinis* cells with those made on fixed and stained preparations. Such comparisons are desirable but have been limited previously to studies of *Saccharomyces* species in which the nucleus of living cells can be seen only during the stationary growth phase. The nucleus of living *R. glutinis* cells is readily visible during the period of bud formation when nuclear division is occurring.

The results show that the two methods employed for studying the nucleus yield comparable information. The nucleus consists of a central optically dense nucleolus surrounded by a layer of chromatin, and the entire structure is enclosed by a membrane. Thus, the gross structure of the *R. glutinis* nucleus is quite similar to that reported for various other fungi (Robinow, 1957, 1961; Bakerspigel, 1957, 1959*a*,*b*). The major point of difference concerns the nuclear membrane, which in many of the light microscopic studies of fungal nuclei has not been observed. The question of whether of not a nuclear membrane exists, however, is best answered by electron microscope studies or ultrathin sections. In all cases where this method has been used on fungi, the nuclear membrane has been clearly visible.

This study shows that the nuclei of *R. glutinis* divide by a process similar to that found for certain yeasts (Hashimoto et al., 1958; Conti and Naylor, 1959) and other fungi (Robinow, 1957; Bakerspigel, 1957, 1959*a*,*b*) rather than in the manner of classical mitosis. Division appears to occur in a direct manner by a process of elongation and constriction. Approximately half of the nucleolus and surrounding chromatin passes into the bud. During the entire process, the nuclear membrane remains intact. No recognizable chromosomes were seen during any of the observed stages of nuclear division. This is in contrast to the recent findings of Robinow (1961), who was able to observe discrete chromosomes in stained preparations of the yeast, *Lipomyces lipofer*, and to follow their behavior during nuclear division. Thus the ability to observe chromosomes may depend not only on the methods employed but also on the type of yeast studied.

Generally, iron alum hematoxylin stains the chromatin material and the nucleolus of the nucleus. In *R. glutinis*, however, only the nucleolus was found to stain, whereas the outer chromatin area had very little affinity for the dye. This suggests that the composition of the nucleic acid of this yeast might be different from that of others. Further cytochemical analysis is required to confirm this view. While the structure of the nuclei in hematoxylin-stained preparations appeared to be similar to that seen with phase contrast microscopy of living cells, chromatin was revealed only in cells stained with HCl-Giemsa or the Feulgen reagent. Only in hematoxylin-stained cells was it possible to follow the division of the nucleolus.

While there seems to be no difficulty in the proper identification of the nucleus in yeast, structural details have not been observed by many. Electron microscope studies have not

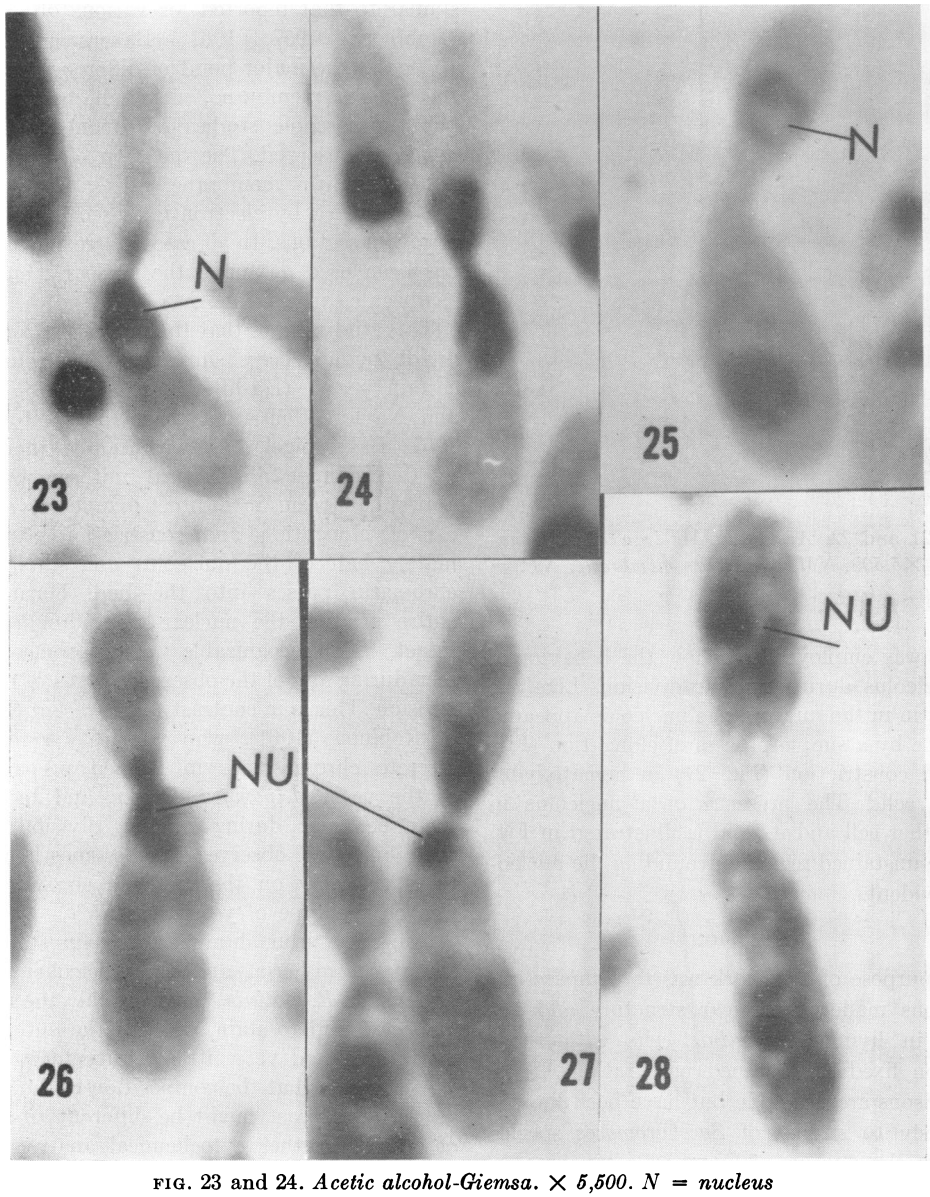

FIG. 23 and 24. *Acetic alcohol-Giemsa.* × *5,500. N = nucleus*
FIG. 25. *Osmic acid vapor-Giemsa.* × *5,500. N = nucleus*
FIG. 26, 27, and 28. *Iodine-formaldehyde-acetic acid and iron alum hematoxylin.* × *5,500. NU = nucleolus*

been of much help in this direction. Recent studies on living cells and stained preparations have definitely enabled the identification of chromocenters and nucleolar equivalents inside the nucleus of certain *Saccharomyces* species (Royan, 1958*a*,*b*; Royan and Subramaniam, 1960), and Robinow (1961) has shown that the nucleus of *L. lipofer* contains defined chromofomes as well as a nucleolus. The present studies of the nucleus in *R. glutinis* have also established the presence of nucleolus and chromatin material inside the nucleus. Discrete chromosomes were not observed during any stage of division of the nucleus, but the presence of granular structures in cells stained by Giemsa, basic suchsin, and Feulgen stains indicates that they

may be tightly packed into the region surrounding the nucleolus.

The absence of vacuoles in young cells of *R. glutinis* suggests that the vacuole is not a permanent structure of the yeast cell, as has been assumed by Mundkur (1954, 1960) and Lindegren, Williams, and McClary (1956).

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