ULTRAVIOLET MICROSCOPY OF BUDDING *SACCHAROMYCES*

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ABSTRACT

McCLARY, DAN O. (Southern Illinois University, Carbondale), WILBERT D. BOWERS, JR., AND GLENDON R. MILLER. Ultraviolet microscopy of budding *Saccharomyces*. J. Bacteriol. **83**:276–283. 1962.—Synchronous cell division was obtained in *Saccharomyces* by transferring starved cells into nutrient medium. Ultraviolet microscopy and Giemsa-stained preparations of these cells showed nuclear division to occur in the mother cell early in the budding process. The divided nucleus passed into the neck between the mother cell and the bud, and either fused together again or the two parts became so closely associated that one continuous dumbbell-shaped body was seen which seemed to divide by constriction. This effect was probably due to the retention of the intact nuclear membrane until nuclear division was otherwise complete.

The nuclear apparatus lies outside the vacuole. The extent to which these bodies function together cannot be determined by the techniques employed in this work.

Parallel experiments on meristematic cells of onion root tips show the reliability of these cytological methods.

The light microscope reveals little of the internal structure of the living, unstained yeast cell. The phase microscope, which produces excellent differentiation in many other organisms, is not adequate for the study of yeast. The yeast cytologist, therefore, has had to rely almost entirely upon cytochemical methods involving the use of various physical and chemical fixatives and dyes for light and electron microscopy, without benefit of adequate controls. The difficulties encountered in the interpretation of such techniques have been discussed by Mundkur (1954, 1960), Hashimoto, Conti, and Naylor (1958, 1959), and others. Certainly the various techniques produce different results, as evidenced by the varying concepts of the yeast nucleus presented in recent publications by contemporary yeast cytologists. Until adequate control observations can be made upon unfixed, living cells, it is unlikely that the structure of the yeast nucleus will be resolved.

Under certain conditions, the ultraviolet microscope may be useful in obtaining information which can aid in the interpretation of observations obtained by other methods. Mundkur (1954) applied ultraviolet microscopy to frozen-dried, sectioned yeast cells but not to living cells. Caspersson and Brandt (1941) and Svihla and Schlenk (1959, 1960) demonstrated the use of the ultraviolet microscope in studying certain interior components of the yeast cell, but they did not observe its nucleus. Sylvén et al. (1959) used ultraviolet microscopy in studying yeast cells synchronized by alternate starvation and growing conditions, but since cytology was not their major concern they devoted little discussion to detailed structure. They did, however, observe the nucleus of starved cells. McClary, Bowers, and Miller (1961) presented a preliminary report on progress made in this laboratory in the study of the nucleus of budding *Saccharomyces* with an ultraviolet microscope illuminated at sublethal intensity. The details of this work are presented in this paper.

MATERIALS AND METHODS

Carbondale tetraploid *Saccharomyces* strain 11294 × 11296 was grown on a medium consisting of glucose, 20 g; $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 2 g; yeast extract, 5 g; and distilled water to 1000 ml. When agar was desired, 20 g of Difco agar was added. Synchronous cell division was obtained for one or two budding cycles by shaking cells overnight in distilled water at 25 C after the manner of Caspersson and Brandt

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(1941), followed by placing them in growth medium (Sylvén et al., 1959).

A Zeiss ultraviolet microscope illuminated with a hydrogen lamp of the Zeiss model PMQ 11 spectrophotometer was used for ultraviolet photography. Visual observation and focusing were accomplished by illumination with the tungsten lamp of the same instrument. Light of any desired wave length between 200 and 2,500 mμ could be selected with the Zeiss M4 Q11 monochrometer. The different focal lengths of visible and ultraviolet light made necessary a few trial-and-error experiments before good pictures were obtainable. By focusing with visible light of 490 mμ, it was found necessary to adjust approximately 3 μ down for focusing at 260 mμ and 1 μ down for 300 mμ. Yeast cells at rest and in various stages of budding were suspended in water on quartz slides, covered with a quartz cover slip, and sealed with Lubriseal. Photographs were taken on Kodak spectroscopic film IIA-0 with a Leitz Makam camera with the glass lenses removed.

Due to the low intensity of illumination of the hydrogen lamp, an exposure time of 7 min was required for best photographic results when a wave length of 260 mμ was used. Considering the lethal effect and morphological changes produced by ultraviolet irradiation of yeast during photography reported by Svihla, Schlenk, and Dainko (1960), it seemed essential that the effects of this illumination be investigated. Svihla et al. (1960) found only slight morphological changes in a minority of cells of *Candida utilis* subjected to 2.1×10^4 ergs per mm^2 of ultraviolet light, and stated that the dose required to produce morphological changes was greater than that required to prevent colony formation when the cells were plated. Pittman and Pedigo (1959) found the LD_{50} dose of the yeast used in these experiments to be 1×10^3 ergs per mm^2. The intensity of illumination at the specimen plane of the microscope used in this study was below the sensitivity of a General Electric ultraviolet intensity meter, but the intensity, measured directly at the slit of the monochrometer some 20 cm farther away, was 0.0086 ergs per mm^2 per sec. The total irradiation of a 7-min exposure was, therefore, less than 3.6 ergs per mm^2. To determine more directly that the illumination used in these experiments would not produce morphological changes, cells in water mounts (5 to 7 μ thick) were photographed at the minimum exposure time and after continuous exposure for 1 hr. No changes could be detected. Although there was no particular concern as to whether or not such irradiated cells were viable, it was determined by plating that viability of a suspension of cells in a quartz cuvette (12 mm outside thickness, 10 mm inside thickness) placed 14.5 cm from the monochrometer slit was not diminished after exposure to the ultraviolet for 1 hr. It was concluded that the illumination used in these studies would affect neither the morphology nor the viability of the yeast cells.

Duplicate samples of all material examined by ultraviolet microscopy were fixed in modified Carnoy solution and stained by the perchloric acid-Giemsa technique (Lindegren, Williams, and McClary, 1956) modified to the extent that hydrolysis was carried out at 60 C for 7 min instead of overnight in the refrigerator at 5 C. These preparations were photographed with the Leitz Ortholux light microscope at 560-mμ, using a Bausch and Lomb interfeıence filter.

The reliability of all cytological techniques was proved by parallel experiments on meristematic cells of onion root tips.

RESULTS

Onion root tip experiments. Unfixed meristematic cells of an onion root tip photographed at 260 mμ are shown in Fig. 1. The nuclei are nearly spherical and contain highly absorbing chromatin threads in a hyaloplasm. In Fig. 2 are seen onion root tip cells fixed in modified Carnoy solution, boiled for 1 min in 50% acetic acid, squashed on a quartz slide in 45% acetic acid, and photographed at 260 mμ. In the dividing cell, the chromosomes are highly absorbent, standing out clearly against a less absorbent cytoplasm. In nondividing cells, the absorbent chromatin is dispersed as granules in the nonabsorbent hyaloplasm. In Fig. 3 the same cells are shown photographed at 300 mμ. In this light, which is absorbed weakly by nucleic acids, the chromosomes are still clearly revealed against the less dense cytoplasm, but appear granular (or, perhaps spiraled) rather than uniformly absorbent. This may be the protein matrix of the chromosomes. These are very comparable with cells fixed and stained according to the perchloric acid-Giemsa technique seen in Fig. 4. The chromosomes and

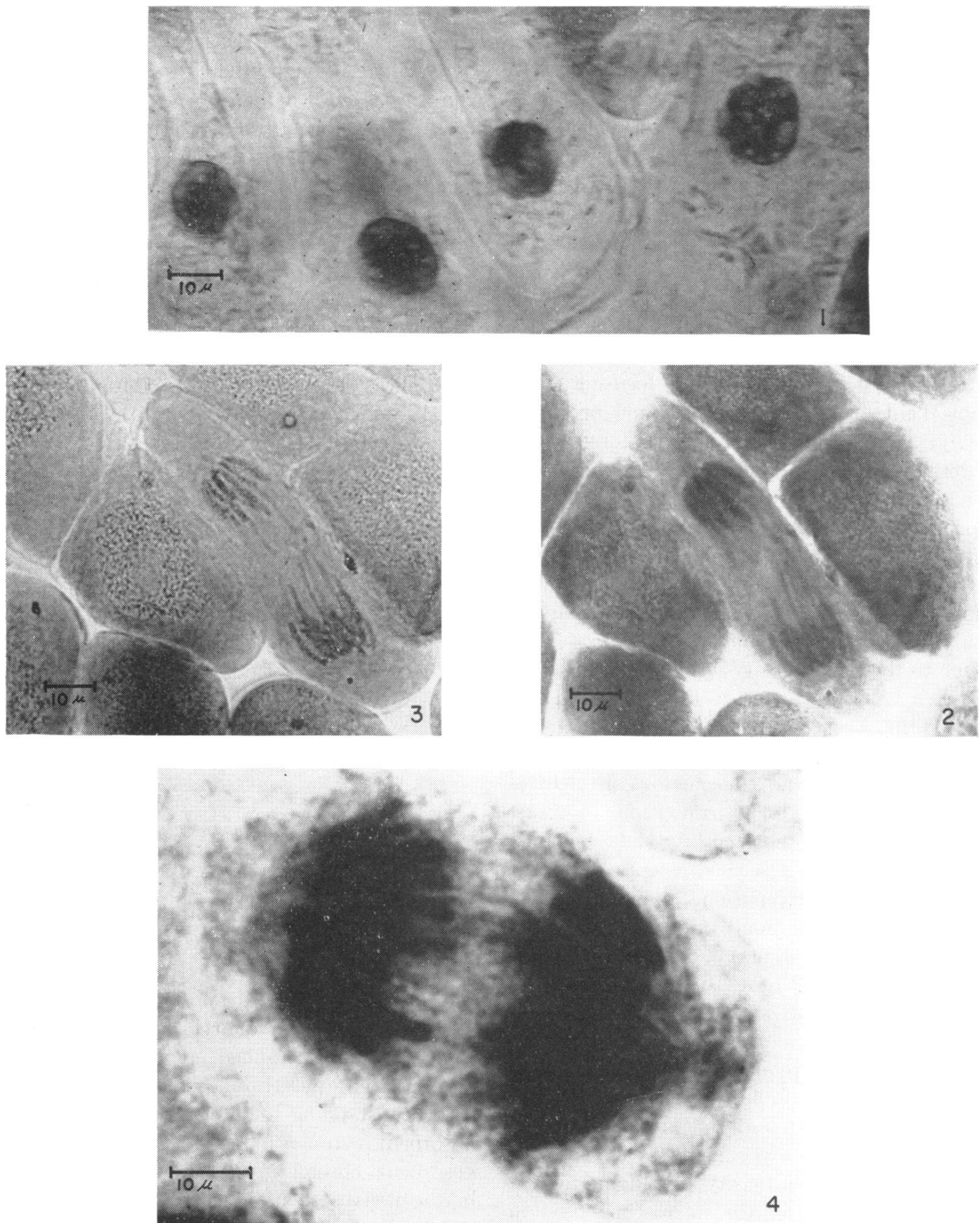


FIG. 1. *Living onion root tip cells photographed at 260 mμ.*

FIG. 2. *Onion root tip cells fixed with the modified Carnoy solution, boiled for 1 min in 50% acetic acid, squashed and mounted in 45% acetic acid, and photographed at 260 mμ. Note that the cell in anaphase, in which chromosomes are so obvious, is an exception. The other cells in interphase have no visible chromosomes.*

FIG. 3. *The same cells shown in Fig. 2, photographed at 300 mμ. Note the granular or, perhaps, spiraled nature of the chromosomes.*

FIG. 4. *An onion root tip cell in anaphase stained by the modified Carnoy-perchloric acid-Giemsa technique. When chromosomes are well dispersed their individual nature is obvious; when they are closely aggregated, as seen near the poles, they appear as a homologous mass.*

spindle fibers of late anaphase are clearly revealed in this photograph.

Yeast cell experiments. Cells starved by shaking in water overnight and photographed at 260 and at 300 mμ are shown in Fig. 5 and 6, respectively. One or sometimes two nonabsorbing vacuoles are seen. A weakly absorbing body is pressed against one side of the vacuole when only one vacuole exists, but it is usually between them when two vacuoles are present, as seen in the elongated cells in Fig. 10. The size and position of this structure enables one to identify it as the spindle (Lindegren et al., 1956; Ywasa and Lindegren, 1959) or as the nucleus (DeLamater, 1950; Mundkur, 1954, 1960; Hashimoto, Conti, and Naylor, 1959; Swaminathan and Ganesan, 1958; and numerous others). Although concepts differ as to whether this object is a hollow vesicle containing the chromatin homogeneously dispersed in nuclear sap, or whether it is a solid spindle upon which the chromatin is partitioned in conventional mitosis, there is a consensus that the chromatin is divided through this structure between the mother cell and the bud during division. It will be referred to hereafter as the "nuclear apparatus" or "nucleus."

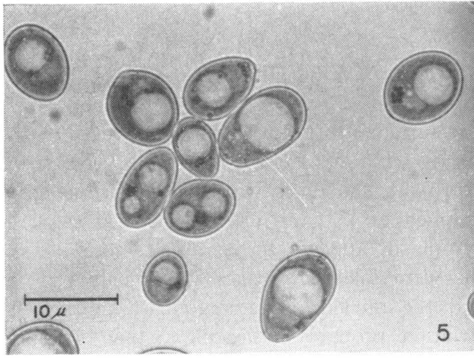
The nuclear apparatus is usually flattened or, in some instances, pressed in by the vacuole, producing a crescent-shaped body when viewed as a plane. In many cases, both the vacuole and the nuclear apparatus are mutually flattened on their adjacent sides. It seems probable that these two bodies pressing outward inside the flexible cell produce its characteristic egg shape, since the smaller body, the nucleus, is always situated toward the small end of the cell. Associated with the nuclear apparatus are one or two clusters of densely absorbing granules. The rest of the cytoplasm is less absorbent and essentially homogeneous except for a few scattered granules. The probable chemical nature and the role of these granules during the growth phase of yeast have been discussed at length by Caspersson and Brandt (1941). Cells starved in 0.07 M phosphate buffer instead of water possessed a more dense cytoplasm and the granules were dispersed; this seems to rule out their being any part of the nuclear apparatus, which was still very weakly absorbent to ultraviolet light but plainly visible (Fig. 7).

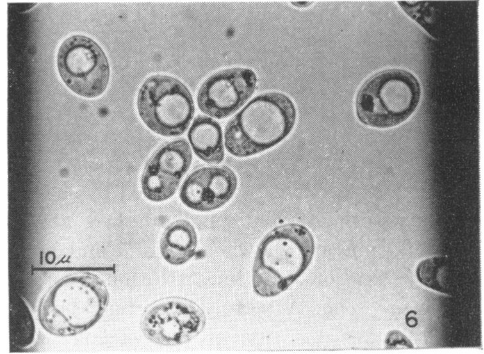
Within a short time after starved cells were introduced into nutrient medium, most of them began budding. Cells which had been starved overnight, shaken in broth for 2 hr, and photographed at 260 mμ are shown in Fig. 8. The cytoplasm appears to be much more dense to the ultraviolet light than that of starved cells, and the nucleus is undergoing a marked constriction, probably indicating a stage of division. In one cell (arrow) one of the lobes of the nucleus appears to be passing into the bud. In a photograph taken a few minutes later (Fig. 9), the clear zones show the lightly absorbing nucleus extending into the bud, at which time it no longer presents the bilobed effect. In cells less advanced, however, the constricted nuclei are still obvious (arrow). In older cultures (16-hr cultures on agar slants), practically all cells were in the resting phase and their nuclei were clearly defined, but a dividing one was seldom seen (Fig. 10). In many respects these cells resemble the starved cells seen in Fig. 5 and 6, even containing the masses of granules which accumulate at the nucleus.

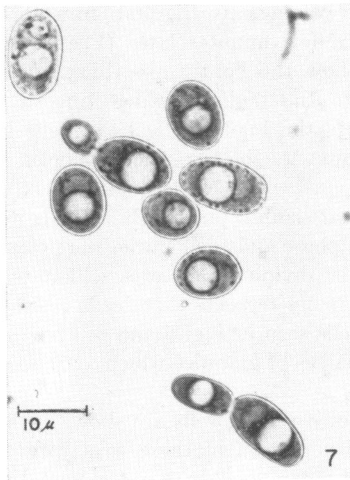
Giemsa-stained resting cells are shown in Fig. 11 and 12. Comparison of these cells with the living cells photographed with ultraviolet light indicates little distortion by this fixation and staining method. The nuclear apparatus occupies the same position in these cells as seen in living cells, and in most cells the vacuole is well preserved. The chromatin, however, is aggregated into irregular masses resembling that of the nondividing nuclei of onion root tip cells (Fig. 1), rather than occupying the nucleus as a homogeneous suspension. Figures 13 and 14 reveal the regular partition of the chromatin into two masses. In one of these cells (Fig. 15 and 15a) the intact nuclear membrane can be seen. Figure 16 depicts a cell in late budding. The chromatin has separated within the mother cell and one portion is extending into the bud.

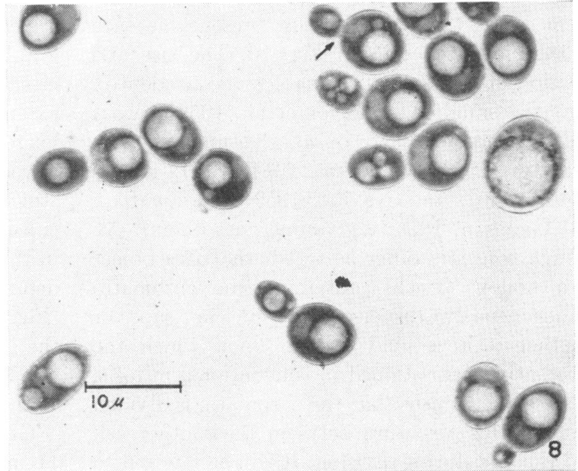
DISCUSSION

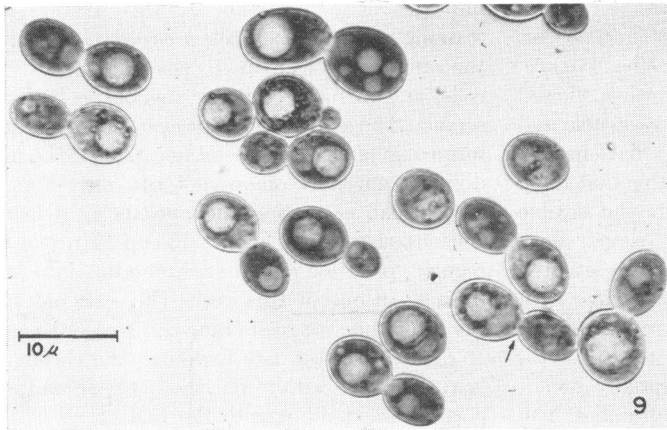
There are essentially three different concepts concerning the nature of the organelle which is called here the "nuclear apparatus" or "nucleus." Lindegren et al. (1956) presented evidence for its being a solid spindle within the vacuole, upon which chromosomes undergo mitotic division. Mundkur (1954, 1960), Hashimoto et al. (1958, 1959), and Necas (1960) found the nucleus to be extravacuolar and devoid of











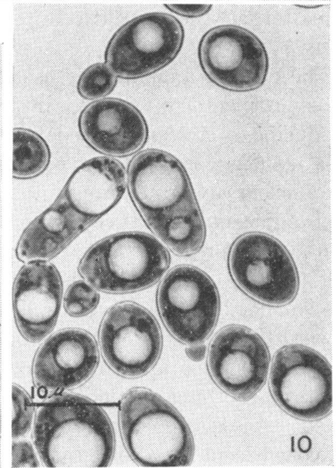


FIG. 5. *Tetraploid yeast cells starved by shaking overnight in distilled water at 25 C and photographed at 260 mμ. The nuclear apparatus is slightly more absorbent to the ultraviolet light than the nonabsorbent vacuole. Note the flattening of both the vacuole and the nucleus on their adjacent sides and the absorbent wall that separates them. The nuclear membrane is not evident. Note the dense masses of absorbent granules accumulated at the nucleus.*

FIG. 6. *The same cells as shown in Fig. 5, photographed at 300 mμ.*

FIG. 7. *Cells shaken overnight in 0.07 M phosphate buffer at 25 C and photographed at 260 mμ. In these cells the granules are dispersed rather than in compact clusters as in water-starved cells.*

FIG. 8. *Cells previously starved in distilled water and photographed at 260 mμ after 2 hr in nutrient medium. Note the constricted nuclei and the homogeneously dense cytoplasm. In the cell denoted by the arrow, the nucleus is moving toward the bud.*

FIG. 9. *Cells from the same preparation a few minutes later. In most cells the nuclei have moved into the neck between the mother cell and the bud and no longer appear constricted. In one cell (arrow) the constricted nucleus is seen. Photographed at 260 mμ.*

FIG. 10. *Cells from a 16-hr slant culture. Except for the ultraviolet density of the cytoplasm, note the similarity between these cells and those shown in Fig. 5. Photographed at 260 mμ.*

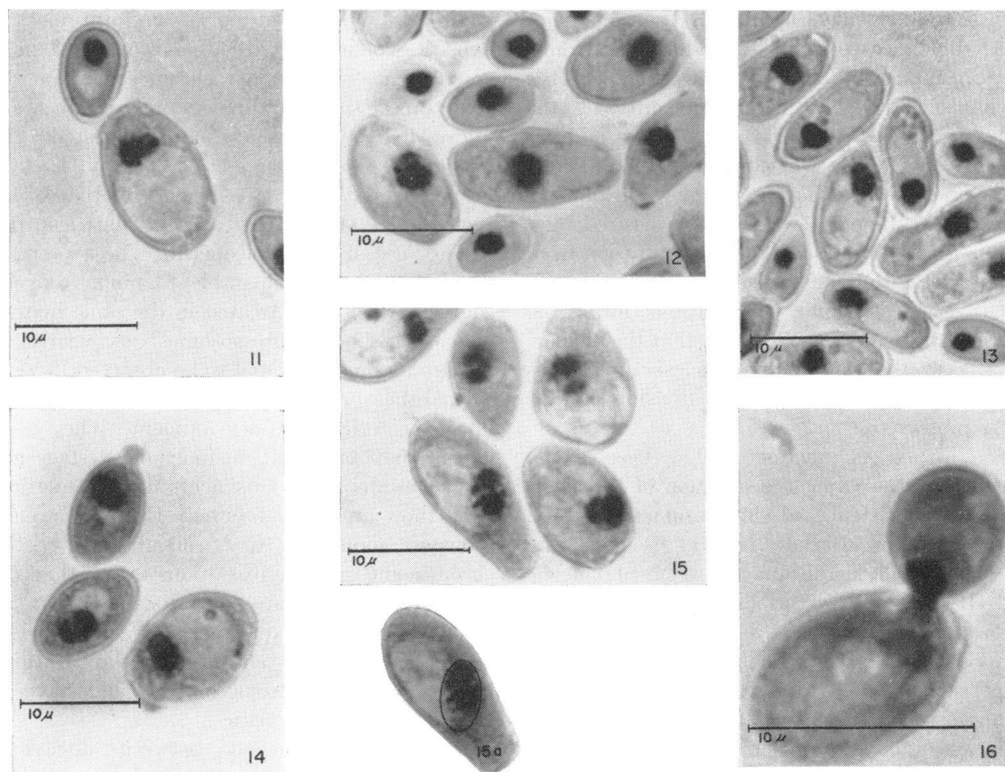


FIG. 11, 12, and 13. *Starved cells stained by the perchloric acid-Giemsa technique. Note the well-preserved vacuoles. The distinct separation of the vacuole and the nuclear apparatus cannot be determined in these preparations, as it can be in the ultraviolet photographs.*

FIG. 14 and 15. *Cells after 1 hr on nutrient media. Note that in every cell the nucleus is divided as in anaphase and that chromosomelike bodies are discernible. In Fig. 15a the nuclear membrane is outlined to mark its position, which can be distinguished in the same cell in Fig. 15.*

FIG. 16. *A budding cell showing the partition of the nuclear apparatus with one nucleus progressing toward the bud while one remains in the mother cell.*

chromosomes within the resolving power of the microscope. They proposed that the nucleus divides amitotically during budding, submicroscopic particles of hereditary substances being separated by simple constriction when the bud has nearly reached the size of the mother cell. DeLamater (1950), Swaminathan and Ganesan (1958), and Ganesan (1959), among others, consider the nucleus to be an extravacuolar body with chromosomes and which divides mitotically. Swaminathan and Ganesan (1958) undertook to resolve the conflicting concepts by proposing a scheme by which mitosis occurred inside the nuclear membrane, which remains intact until it extends into the bud and finally separates by a constriction process resembling amitosis.

Ultraviolet microscopy seems to offer a solution to some of these differences. The nuclear apparatus apparently lies outside the vacuole. This is indicated by the ultraviolet-absorbent wall, which completely surrounds the vacuole separating it from the nuclear apparatus, and by the fact that both of these structures are mutually distorted on their adjacent sides, as would be the case when two flexible objects, such as balloons, are pressed together. This, however, need not indicate that they do not function together as the nucleus. Under certain conditions, the nucleolus and chromosomes appear in the vacuole (Lindegren and Rafalko, 1950; Lindegren, McClary, and Williams, 1955) as do other substances such as metaphosphate (Lindegren et al., 1955) and S-adenosylmethionine

(Svihla and Schlenk, 1959, 1960). The function of these substances under the conditions in which they appear are still, however, uncertain, as is the mechanism by which they are transmitted to the buds. Svihla and Schlenk could observe no division of the vacuole when S-adenosylmethionine was transferred to the bud under conditions in which the compound was not synthesized, and proposed that the transfer was by diffusion from the vacuole into the cytoplasm of the mother cell and thence to the bud. Observations during the course of this study also indicate that the vacuole appears in the bud before nuclear division is complete and does not involve division of that of the mother cell.

The ultraviolet microscope has been of no assistance in resolving the question of the existence or nonexistence of chromosomes in yeast. Caspersson and Brandt (1941) discussed the difficulty in distinguishing between ribonucleic acid (RNA)- and deoxyribonucleic acid (DNA)-containing components of the yeast cell by ultraviolet microscopy, pointing out the high proportion of RNA to DNA and the fact that both substances have similar absorption spectra. They were unable to detect bodies corresponding to those found in their Feulgen-stained controls, but they stated that with certain adjustments a "dent" could be observed in the vacuole which looked as if it were made by a round body that might be analogous to the "euchromatin" of cells of higher organisms.

The low concentration of DNA presents the same difficulty in observing the yeast nucleus stained by the Feulgen technique as is encountered with the ultraviolet microscope. Although this stain is an excellent nuclear stain for higher organisms, it has not resolved controversies concerning the nature of the yeast nucleus. Several authors, using this method or similar ones based on the same principles, have described chromosomes; others, using the same methods, have found that the nucleus is a homogeneous structure. Few, however, have been able to obtain photographs suitable for publication; more often, other nuclear stains, such as Giemsa, hematoxylin, or pyronin, were used, with statements then being made of having adequately supported them with Feulgen-stained controls. It is, therefore, evident that the Feulgen technique is not a suitable one for the yeast nucleus.

Most electron micrographs of the yeast cell show the nucleus to be of uniform electron density. Yotsuyanagi (1960), however, produced convincing evidence for chromosomes in this structure. Using ultrafine sections of *Saccharomyces* fixed in permanganate, digested with ribonuclease, and treated with uranyl acetate, he increased the electron density of the chromosomes so that they appeared in sharp contrast to the rest of the nucleoplasm. These preparations were very comparable to onion root tip controls which were treated in the same manner and which showed chromosomes very clearly.

Chromosomes may fail to be observed in yeast as in other organisms if they are examined in improper stages of development. They may appear only briefly at an unexpected stage and in such a minority in unsynchronized populations that they are not observed. Even in rapidly growing onion root tips, cells bearing visible chromosomes are the exceptions rather than the rule, although they are so obvious that they are recognized without question (Fig. 2–4). Based on the majority of cells in these preparations, those containing chromosomes would have to be considered irregularities.

The close agreement between ultraviolet microscopy and the results obtained with the perchloric acid-Giemsa technique on onion tip controls inspires a great degree of confidence in the latter as a reliable cytological stain. Synchronized cultures in early budding stained by this method reveal regularly figures resembling the anaphase stage of mitosis (Fig. 13–16). This, so far as division into two components is concerned, is confirmed by ultraviolet microscopy of living cells (Fig. 5–9). When it is noted that these are not isolated, exceptional cells but are clusters representative of an entire culture, it is difficult to consider them as artifacts of chemical fixation and dying. The importance of the age and synchronization of the culture is illustrated in Fig. 10. This ultraviolet photograph of cells from a 16-hr slant, which may ordinarily be considered a young culture, shows no dividing nuclei and the cells in general resemble those from a starved culture.

Vegetative division in *Saccharomyces* seems analogous to that of the yeast *Lipomyces lipofera* described by Ganesan and Roberts (1959) and Robinow (1961). Robinow described an early division of the chromosomes by mitosis, which was followed by a fusing together of the chromosomes into a continuous bar that divided be-

tween the mother cell and the bud by constriction. This process is indicated in *Saccharomyces* by Fig. 8 and 9. The marked constrictions of the nuclei, which are obvious in nearly every cell in Fig. 8, are no longer apparent in nuclei passing into the buds somewhat later (Fig. 9).

Although the nuclear membrane is not revealed by the ultraviolet microscope and is not very conspicuous in most stained preparations, its extension into the bud, and its eventual separation by constriction when the bud approaches the size of the mother cell, seems clearly established by numerous electron micrographs (Hashimoto et al., 1959; Mundkur, 1960; and others).

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