THE NUCLEUS OF SACCHAROMYCES BAYANUS

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INTRODUCTION

YEAST can be grown in a variety of media. The crisp nuclear details observed in living cells aged in barley malt wort (Royan, 1958 a, b) could rarely be seen in those from glucose-peptone-yeast extract medium. The nuclear membrane of cells grown in Ragi (Eleusine coracana) malt wort appeared thicker as compared to those from barley malt wort (Royan-Subramaniam, unpublished).

The structural details of the yeast nucleus as seen under ordinary, phase contrast and dark ground illumination were published some years ago (Royan and Subramaniam, 1956). The nuclear organelles themselves have been categorized using fixatives and stains (Royan, 1958 c; Subramaniam, 1960). The above observations were also confirmed by vital staining and fluorescence microscopy (Royan and Subramaniam, 1960). The statement that the "phase microscope which produces excellent differentiation in many other organisms is not adequate for the study of Yeast" and that "until adequate control observations can be made upon unfixed living cells, it is unlikely that the structure of the Yeast nucleus will be resolved" (McClary, Bowers and Miller, 1962) appeared therefore surprising. What is not still realized is the necessity for an exploration of the effect of the composition of the media and the aging of cells on the visibility as well as the architecture of the nucleus in living yeast cells.

The structure of the nucleus of Saccharomyces bayanus attracted our attention since it was from 1-6-hour old shaken cultures of this species that Lindegren and Rafalko (1950) offered evidence in support of their contention that the vacuole of yeast is its nucleus. Since they could not demonstrate in living material many of the details seen on staining, it became interesting to elucidate whether the details of the real nucleus (Subramaniam, 1960) could be photographed in the living cells.

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1. Living Cells

The strain of *Saccharomyces bayanus* investigated was received through the courtesy of Dr. Wickerham. They were grown in barley malt wort of S.G. 1.020 and pH 4.6-4.8. The majority of the cells in 16-24 hour cultures had homogeneous cytoplasm and prominent vacuoles. Almost equal numbers of uni- and bi-vacuolate cells were observed in 3-day cultures. Only in 5% of the cells of both types could the nucleus be located. The cytoplasmic granules which began to appear on the 3rd day became increasingly prominent on aging. This necessitated a limitation of the observations to cells from 72-96-hour cultures in which the nuclear details were not obscured by the cytoplasmic granules.

*Phase Contrast.*—In Photos 1-3 the vesicular nucleus showing denser structures in its matrix is seen in the band of cytoplasm separating the two vacuoles. Only in some regions is the nuclear membrane clear. The intra-nuclear structures may be apposed to the inner surface of the nuclear membrane (Photo 1) or lie free in the matrix (Photos 2 and 3).

*Bright Field.*—Photos 4 and 5 illustrate the orientation of the nucleus in bi- and uni-vacuolate cells. In both, the nuclear membrane appears well defined and the matrix homogeneous. The uneven thickening of the membrane is more pronounced in Photo 6. The intra-nuclear structures lying apposed to the membrane appear to be responsible for these thickenings. Support for such a belief is offered by the three spherical grains, two in contact with the nuclear membrane and the third lying free, in Photo 6. The dense intra-nuclear matter had often no well-defined outlines (Photos 7 and 8). Crescent-shaped thickening of the nuclear wall was not uncommon (Photos 9 and 10).

A number of intra-nuclear bodies differing in size as well as shape are seen in close apposition to the nuclear membrane in Photo 11. Unless the nuclear membrane is clear, these may be mistaken for cytoplasmic granules. The clear thin membrane which overlaps the vacuole (indicated by an arrow in Photo 11) would confirm that these are lying in the nuclear matrix. The nucleus was occasionally visible in the mother as well as the bud (Photo 12).

*Dark Ground Illumination.*—The presence of a limiting membrane for the vacuoles was surmised by Lindegren and Rafalko (1950) by the regular contour of the vacuoles and not from actual observations on living cells. The vacuolar membrane is an impermanent structure (Aswathanarayana, 1956, 1958) and becomes visible only on aging the cells. The luminous vacuolar
boundaries in cells from 72–96 hour cultures are illustrated in Photos 13 and 14. Contrary to Lindegren’s (1949) contention the regularity of their outline in bi-vacuolate cells indicate that they are not interconnected.

The nucleus was seen under dark ground illumination only in favourable instances. The vacuoles and the nucleus were not in the same plane in Photos 15 and 16. The vacuolar borders were, therefore, out of focus in the above photos. The irregularly thickened appearance of the nuclear membrane is clear in Photo 16.

2. Fixation

Life-like preservation was obtained with iodine-formaldehyde-acetic acid (I.F.A.—Gram’s iodine 22·5 ml., formaldehyde 6 ml., acetic acid 1·5 ml., distilled water 22·5 ml.; Royan, 1956, 1958 a, b; Aswathanarayana and Subramaniam, 1958). Fixation in a test-tube led to a clumping of the cells and precluded getting smears one cell thick, whether the material was handled from the fixative itself, or suspended in 70% alcohol or distilled water. Necessarily, therefore, fresh yeast was smeared on a slide, exposed to the vapour of the fixative to prevent the loosening of the cells during processing and then plunged into a jar of the fixative. The time of fixation was one hour.

Since a comparison of the living with fixed and stained preparations gave the impression that the structure of the nucleus was the same even when it was not clearly visible in the living condition, such a possibility was explored by a study of the reactions of a few living cells mounted under a cover-slip to irrigation with the fixative. Extensive trials in this direction gave only indifferent results.

The procedure adopted was to mount a few cells in a drop of the medium in which they had grown and to draw the fixative continuously from one side of the coverslip to the other using filter-paper strips. Since during this process the fixative gets diluted by the medium in which the cells are mounted, it is difficult to be sure of the concentration of the fixative when it reached the cells. Experience on smears indicated that the concentration of the fixative used is an important factor (Gatenby and Cowdry, 1928; Johansen, 1940; Darlington and La Cour, 1950; Baker, 1955).

Because only small droplets could be added at a time, the movement of the fixative under the coverslip was rather slow. It does not act simultaneously on all sides of the cells under observation. If the fixative is added in larger droplets, the cells float away. In most cases, there is a sudden
contraction of the cell when the fixative reaches it as also a sudden granulation of the cytoplasm. Gram's iodine was comparatively better in this respect. These are abnormal phenomena when compared to fixation of material as smears or in a test-tube. The exposure of only portions of the cells to the action of the diluted fixative and the consequent absence of a sudden killing action and the effect of bright illumination necessary for photography were factors beyond the control of the experimenter.

Photo 17 is of a living cell in which the nucleus though structureless was denser. On irrigation with Gram's iodine for 15 min., the nucleus appeared as a vesicle with a well-defined membrane (Photo 18). These observations, when extended to living cells in which the nucleus was either indistinct or appeared as a clear area, revealed on irrigation with Gram's iodine, nuclear details comparable to that seen in ideal examples of living nuclei. The clear area in Photo 19 is the nucleus. Ten minutes after the commencement of irrigation, it was seen to have a well-defined thin nuclear membrane with cytoplasmic granules lying close to it (Photo 20). Indications of the presence of a nuclear membrane are seen in the living cell illustrated in Photo 21. It appears better defined than those in Photos 17 and 19. On irrigation with Gram's iodine for 15 min., the nuclear boundary became well defined with indications of formed structures within the nucleus. A comparison of Photos 23 and 24 would show the overall reduction in size, of a bi-vacuolate living cell with a clearly visible nucleus, on irrigation with Gram's iodine for 25 min. These experiments suggest that the nucleus has the same architecture even when it is invisible in the living condition.

The difference between fixation of a few cells under a coverslip (Photos 17-24) and en masse in a test-tube or as a smear is rather pronounced. Photos 25-29 are of cells fixed as a smear in I.F.A., and illustrate the details of variations observed in the nuclei.

3. Staining

A. Haematoxylin.—The smears were hydrolysed in N HCl at 60° C. for 7–8 min. or in 10% perchloric acid at 28° C. for 3–4 hours and then stained with haematoxylin (Aswathanarayana and Subramaniám, 1958). The nucleus is homogeneous in Photo 30. In Photo 31 the nuclear membrane is visible in the crescentic unstained region of the nucleus. The intra-nuclear structures vary in number, size and disposition (Photos 31–34). Two stained masses are separated by an unstained band in Photo 32, while two spherical masses are lying close together in Photo 33. The nucleus in Photo 34 has a large irregular crescentic patch with a small stained area separated from it.
Attention is invited to the distinct membrane delimiting the nucleus from the cytoplasm (Photos 31–34).

**B. Feulgen Technique.**—The procedure adopted was the same as described earlier (Aswathanarayana and Subramaniam, 1958). Lindegren and Rafalko (1950) found the staining limited to regions of the organelles identified by them as centrosomes. Only in rare instances did they observe the entire organelle stained. In unhydrolysed control slides kept for the same duration in the Schiff's reagent and handled simultaneously with the hydrolysed ones, no staining was observed either in the nucleus or the vacuole (Photo 35). The cells of *S. bayanus* exhibit, therefore, no plasmal reaction.

The Feulgen-positive substance is seen to occupy only a small area of the nucleus in Photos 36–41. Apart from the differences in size (compare Photos 36, 37 with 38), the orientation of the stained regions inside the nucleus was also variable (see p. 173, Lindegren and Rafalko, 1950). The stained mass is lying free in Photos 36 and 39 but in contact with the nuclear membrane in Photos 37, 38 and 40. The unstained nuclear membrane is clear even under ordinary illumination (Photos 38–40) and examination under phase contrast did not improve its clarity (Photo 41). The vacuole was uniformly Feulgen-negative.

**C. Staining with Giemsa's Solution.**—Smears hydrolysed either in N HCl at 60 °C. for 8–12 min. or with perchloric acid at 28 °C. for 16 hours were stained with Giemsa (Gurr, Michrome-brand Special, 4 ml., tap-water 20 ml., distilled water 20 ml.) for 60–120 min., destained with 20% alcohol, washed well in water and then mounted under a coverslip. The illustrations presented are from such water mounts. The cytoplasm and the nuclear membrane were light pink while the intra-nuclear structures were reddish. Hydrolysis in N HCl produces a brighter staining of the intra-nuclear structures. Traces of the “nucleolar equivalent” (Royan, 1958 c) were seen occasionally in some cells.

Photo 42 is interesting in that the nuclear membrane overlaps the vacuoles lying on either side of it. The stained mass was forked, a feature not clearly brought out by the photograph. Attention is invited to the absence of any stained structures in the vacuoles. In the cell shown in Photo 43, the nuclear membrane was not distinct and the red-stained area had a hemispherical shape. No other details were visible. The configuration of the nuclei of the mother and bud often differ. In the budding cell presented as Photo 44, the nuclei of the mother and bud lie in different planes rendering difficult their demonstration in a single photograph. Though the nuclear membrane
The Nucleus of Saccharomyces bayanus was clear in both, the nuclear details are very clear only in the mother cell (Photo 44).

A comparison of haematoxylin and Feulgen preparations indicated that the nucleus contained also Feulgen-negative organelles which have necessarily to be considered as "nucleolar equivalents" (Royan, 1958 c). Such a differentiation is possible only if the unhydrolysed cells are stained with Giemsa (Royan, 1958 c).

Therefore unhydrolysed smears were transferred to Giemsa stain and examined periodically. An exposure for two hours to the stain was found generally satisfactory. The staining was often erratic. The real difficulty experienced was the quick removal of the red colour by the 20% alcohol used for differentiation. Control of the time of staining minimises this difficulty. In optimally stained cells, the nuclear membrane is blue, the chromocentres red and the nucleolar equivalents blue (Photos 45 and 46). The nuclear membrane in Photo 45 overlaps the vacuoles lying on either side of the nucleus. In Photo 46 the chromocentre is rod-shaped and the nucleolar equivalent a hemispherical mass plastered on to the inner surface of the membrane. The deeply stained crescentic nucleolar equivalent is in focus.

DISCUSSION

The vacuolar and nuclear membranes as well as organelles in the nuclear matrix could be recognized and photographed in living cells from 72-96-hour cultures, under suitable types of illumination (Photos 1–16). One or more ‘dancing bodies’ alone were visible in the vacuoles of some living cells of all ages, when examined under phase contrast and dark ground illumination. The superposability of haematoxylin pictures (Photos 30–34) on those of living cells emphasize that the vacuole is not the nucleus. It is only a cytoplasmic inclusion.

The cyclical relationship in the reaction of the vacuole and the “centrosome” (= spindle with chromatin, Lindegren and Townsend, 1954; Spindle, Lindegren, Williams and McClary, 1956; Nucleus, McClary, Bowers and Miller, 1962; Spindle Reservoir, Lindegren, 1962; Nucleus, Hirano, 1962) to the Feulgen stain (Lindegren and Rafalko, 1950) did not find a confirmation in the present investigation. The Feulgen-negative nature of the vacuole and the occurrence of the Feulgen-positive material within the area bounded by the nuclear membrane would imply that the body identified as the “centrosome” by Lindegren and Rafalko (1950) is the real nucleus.
The organization of the yeast nucleus appears similar in different species and strains (Subramaniam, 1960; Royan and Subramaniam, 1960). In structural details the nucleus of *S. bayanus* conforms to those of higher organisms (Subramaniam, Royan, Thyagarajan, Aswathanarayana and Subramanyam, 1959; Subramanyam, 1960; Yotsuyanagi, 1960).

**SUMMARY**

The extra-vacuolar nucleus is visible in a small percentage of living cells from 72–96 hour wort cultures. The vacuoles show a luminous boundary under dark ground illumination. The details observed in living nuclei could be stained with haematoxylin after fixation in iodine-formaldehyde-acetic acid mixture. The Feulgen-negative nature of the vacuole and the limitation of the Feulgen-positive material to the area bounded by the nuclear membrane would imply that the ‘centrosome’ described by Lindegren and Rafalko (1950) is the real nucleus. The nucleus of *S. bayanus* conforms in its structure to those of higher organisms.

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**DESCRIPTION OF PHOTOMICROGRAPHS**

**PLATE XXIII**

*Photos 1–12. Living Cells from 72–96 hour cultures, × ca. 4,000.*

**PHOTOS 1–3.** Phase Contrast. The nucleus is lying in the cytoplasmic band between the vacuoles. The nuclear membrane encloses formed structures.

**PHOTOS 4–12.** Bright Field.

**PHOTOS 4 & 5.** The nuclear membrane delimits a homogeneous matrix. Bi- and uni-vacuolate cells.

**PHOTO 6.** The nuclear membrane is uneven in thickness. There is a grain in the nuclear matrix.

**PHOTO 7.** Attached to the nuclear wall is a dense intra-nuclear structure.

**PHOTO 8.** The nucleus has a membrane of almost uniform thickness. Bi-vacuolate cell.

**PHOTOS 9 & 10.** A crescentic structure lies apposed to the inner surface of the nuclear membrane.

**PHOTO 11.** Bodies of differing sizes lie in close apposition to the nuclear membrane.

**PHOTO 12.** Nuclei of the mother and bud.

**PLATE XXIV**

*Photos 13–16. Living Cells. Dark ground illumination*

*Photos 17–24. Action of Fixative*

**PHOTOS 13 & 14.** The vacuoles of bi-vacuolate cells have distinct luminous boundaries.

**PHOTOS 15 & 16.** The nucleus is lying in the band of cytoplasm between the vacuoles.

**PHOTOS 17–24.** Irrigations of living cells mounted under a coverslip with Gram's iodine. × ca. 3,000.
PHOTOS 17 & 18. A living cell (Photo 17) in which the nucleus becomes clearer after fixation (Photo 18).

PHOTOS 19 & 20. Another example of a living cell (Photo 19) in which the nucleus is revealed by fixation.

PHOTOS 21 & 22. The structure of the nucleus becomes clear on irrigation with the fixative (Photo 22) of a living cell (Photo 21).


PLATE XXV

Photos 25-46. Cells fixed in iodine-formaldehyde-acetic acid mixture for 60 min.

PHOTOS 25-29. Cells from the fixative. The nucleus and the vacuole are well preserved, \( \times ca. 4,000 \).

PHOTOS 30-34. Stained with Heidenhain’s haematoxylin, \( \times ca. 5,000 \).

PHOTOS 30, 32 & 33. Hydrolysed in NHCl at 60°C for 8 min.

PHOTOS 31 & 34. Hydrolysed in 10% perchloric acid at 28°C for 4 hours.

PHOTO 30. The nucleus is stained homogeneously.

PHOTO 31. The nuclear membrane is seen in the crescentic unstained region.

PHOTO 32. The two stained structures inside the nucleus are separated by an unstained band.

PHOTO 33. Two stained spherical masses are lying close together inside the nucleus.

PHOTO 34. The nuclear membrane encloses an irregular crescentic patch and a small grain.

PHOTOS 35-41. Cells stained by the Feulgen Technique, \( \times ca. 5,000 \).

PHOTO 35. Unhydrolysed cell stained to show the absence of any plasmal reaction.

PHOTOS 36-38 & 41. Hydrolysed in NHCl at 60°C for 9 min.

PHOTOS 39 & 40. Stored in perchloric acid at 28°C for 6 hours.

PHOTOS 36-40. The Feulgen-positive substance shows differences in size as well as orientation.

PHOTO 41. Nuclear membrane encloses the Feulgen-positive area. Phase Contrast.

PHOTOS 42-46. Cells stained with Giemsa. Water mounts, \( \times ca. 5,000 \).

PHOTO 42. The nucleus lies over a vacuole in a bi-vacuolate cell. The stained structure is bounded by the nuclear membrane.

PHOTO 43. The stained area has a hemispherical shape.

PHOTO 44. The thin nuclear membrane is seen enclosing stained areas in the mother cell.

PHOTOS 45 & 46. Unhydrolysed cells. The nuclear structures are stained differentially. The chromocentre (R) is red while the nucleolar equivalent (B) is blue.

\( N \)—Nucleus; \( V.M. \)—Vacuolar Membrane.
Figs. 1-12
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Figs. 13-24
Figs. 25-46