

# THE NUCLEUS OF THE LIVING ZYGOTE OF *SACCHAROMYCES CARLSBERGENSIS*

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## INTRODUCTION

THE recent attempts to elucidate the structure of the resting nucleus of yeast using the living cell as a "standard" (Royan and Subramaniam, 1956; Royan, 1956 *a, b, c*; Muller, 1956; Thyagarajan and Subramaniam, 1957) originate from the general dissatisfaction felt by investigators of the limitations of orthodox fixing and staining procedures when applied to yeast. The invisibility of the nucleus under most physiological conditions had precluded so far an accurate evaluation of the reliability of the various cytological procedures for a study of the resting nucleus.

To fill up this important lacuna in our knowledge, a programme of active search for the living nucleus was initiated in this laboratory three years back and cultures of various species and strains grown under differing physiological conditions were examined systematically with a phase contrast microscope. The nucleus was visible in 40–60% of the cells from 96–120-hour wort cultures of a strain of *Saccharomyces cerevisiæ* grown at 24–26° C. Micrographs taken under bright field, phase contrast and dark ground illumination (Royan and Subramaniam, 1956; Royan, 1956 *a, b, c*) were presented and it was shown that the nucleus could be kept under observation during the cytological procedures like fixation, hydrolysis and staining (Royan, 1956 *a*). A fair idea of the reaction of the nucleus to a variety of fixatives and stains is now available (Royan, unpublished).

In *Saccharomyces carlsbergensis* the nucleus could be seen in 5–10% of the living cells from 160–190-hour wort cultures (Thyagarajan and Subramaniam, 1957). In *S. cerevisiæ* as well as in *S. carlsbergensis* the nucleus if once located under phase contrast could be observed under ordinary illumination. Muller (1956) studied the role of the refractive index of the media in rendering visible the nucleus in yeast cells in which it is normally invisible. A comparison of the photomicrographs presented by Muller (1956) of cells of *S. carlsbergensis* mounted in a medium of suitable refractive index, with those obtained by us (Thyagarajan and Subramaniam, 1957) revealed that more nuclear details could be observed in 7-day wort cultures.

There are no previous reports on the nucleus of living zygotes of yeast. The zygote of *Saccharomyces carlsbergensis* possesses a very desirable characteristic. The shape assumed at the time of its origin by fusion of two spores is retained even after the zygote had produced a few buds. Since the nucleus becomes visible in the vegetative cells only when the culture has aged, the possibility of the nucleus being visible in those zygotes retaining their characteristic shape 6-7 days after the introduction of the spores into fresh wort was explored. The nucleus is clearly visible in 40-50% of the zygotes in 7-day cultures.

#### HISTORICAL RESUMÉ

Reports on the zygote nuclei based on stained preparations are rather few. From material fixed in Perenyi's fluid and stained with hæmatoxylin, Guilliermond describes the zygote nuclei of *Saccharomyces ellipsoideus* var. *Johannisberg* II (1910) and *Schizosaccharomyces octosporus* (1917) as vesicular and possessing a nuclear membrane, a nucleolus and often a chromatic reticulum.

Winge (1935) employed two different procedures for the study of the zygote nuclei of *Saccharomyces ellipsoideus* var. *Johannisberg*. For staining with hæmatoxylin the fixation was in Muller's fluid, while for the Feulgen technique it was in chloroform. The nucleus described and illustrated by him is a solid structure lacking the finer details reported by Guilliermond (1910).

Badian (1937) using the Giemsa stain observed two chromosomes in the spores as well as the zygotes of *Saccharomyces cerevisiæ*. He did not observe a nuclear membrane.

Lietz (1951) does not refer to the existence of a nuclear membrane in zygote nuclei though he records its presence in vegetative cells. Widra and DeLamater (1955) investigated *Schizosaccharomyces octosporus* but their description of the zygote nucleus is at variance with that of Guilliermond (1917). They surmise the existence of a nuclear membrane even though they could not reveal the nuclear details illustrated by Guilliermond.

#### EXPERIMENTAL PROCEDURES

We are indebted to Dr. L. J. Wickerham for the gift of the strain of *Saccharomyces carlsbergensis*. Cells from 16-hour barley malt wort (S.G. 1.020; pH 4.6-4.8) cultures were streaked on wort agar slopes. When growth had proceeded for 24 hours material was transferred to acetate agar (Sodium acetate 0.4%; Agar 1.5%, cf. Fowell, 1952) slants and examined periodically. Spores begin to appear 24-36 hours after transfer to acetate

and since 10-day old slants contained a large number of spores, they were used as the source for the germination studies.

Not all cells sporulate on acetate agar and naturally the inoculum introduced into wort would contain vegetative cells also. These were killed by keeping the tubes in water at 55° C. for five minutes. Due to the heat treatment zygotes begin to appear only after 16 hours after transfer to wort and viable spores give rise to zygotes within 48 hours.

When the nuclei become visible in the zygotes on the 7th day, the cells were mounted in a drop of the medium in which they had grown and two moist filter-paper strips were kept on either side of the square coverslip to flatten the cells uniformly and prevent their movement. They were photographed with a Leica attachment on Kodak 'Microfile' film. The negatives were later enlarged for reproduction.

#### OBSERVATIONS

##### 1. *Living Cells*

Photo 1 is that of a zygote observed three days after the introduction of the spores into wort. The cytoplasm appears dense, the two vacuoles are clear, but the nucleus cannot be located in the living cell. It would seem that any region of a zygote can produce a bud (Photos 1-3 and 5-10). In Photos 9 and 10 the bud has originated from the bridge-like waist of the zygote. Attention is invited to the zygote in micrograph 5 which has two buds of different ages still attached to it (compare with Fig. 9 A, Lietz, 1951).

With the aging of the culture the cytoplasm slowly loses its opacity under phase contrast and the nucleus becomes gradually visible (compare Photo 1 with Photos 5-10). On the 7th day it could be photographed in favourable examples even under ordinary illumination (Photos 2, 3 and 4). It has a conventional structure in that the nuclear membrane (NM, Photos 2, 3 and 4) which delimits it from the cytoplasm encloses visible structures (Royan and Subramaniam, 1956; Royan, 1956 *a*). A crescentic denser area attached to the inner surface of the nuclear membrane is visible in Photo 3 while a grain is present in Photo 4.

The phase contrast microscope reveals more details. The structure of the nucleus shows considerable variation. The nucleus in Photo 6 has the appearance of a dense ring enclosing in its clear interior a distinct grain. The thickness of the nuclear periphery is not uniform in the zygote illustrated in micrograph 7. As in the previous instance there is a distinct granule in the middle of the clear area. That the thickened appearance of the nuclear periphery is due to formed structures plastered on to the inner surface of the

nuclear membrane would become evident from Photo 8. The nuclear membrane (NM) is thin near the clear interior of the nucleus. Half the nucleus is partially opaque (Photo 8) reminding one of the crescentic veil-like area in micrograph 3. There is a dense sickle-shaped mass plastered on to the inner surface of the nuclear membrane. A grain is lying free inside the nucleus.

Since the zygotes had buds attached to them, the culture was scanned for instances where the nuclei could be observed simultaneously in a zygote and its bud. Micrographs 9 and 10 illustrate such a rare example. The two nuclei were not at the same optical plane and hence two photographs are presented to illustrate their orientation as well as structure. The cytoplasm was optically denser than in the cells shown in Photos 6, 7 and 8 with the consequence that the nucleus does not show good contrast as in the preceding illustrations. The nucleus of the bud has the same structure as that of the zygote. What is more, it resembles in structure the living nucleus of the vegetative cells illustrated earlier (Thyagarajan and Subramaniam, 1957).

## 2. *Fixed and Stained Preparations*

Two investigators (Guilliermond, 1910; Winge, 1935) who studied the nucleus of *Saccharomyces ellipsoideus* var. *Johannisberg* have presented differing descriptions of the structure of the nucleus. The details observable in the nucleus of the living zygotes, illustrated by photographs for the first time in this paper, if used as a basis for an evaluation of the published results would suggest that the fixation and staining procedures employed by Guilliermond gave a picture approaching that of the living nucleus. Guilliermond's suggestion that the nuclei of the zygotes and vegetative cells resemble each other in that they are delimited from the cytoplasm by a nuclear membrane is borne out by micrographs 9 and 10 illustrating such a membrane in the living nuclei of a zygote and its bud (*cf.* Royan, 1956 *a, b, c*; Thyagarajan and Subramaniam, 1957).

The earlier descriptions of nuclear structure (Guilliermond, 1910; Winge, 1935) are based on fixed and stained preparations owing to the invisibility of the nucleus in the living zygote. Naturally later investigators agree with Guilliermond or Winge depending on the technique employed and the results obtained by them (Ganesan, 1956). The way one handles the staining procedures becomes the criterion for evaluation in the absence of a "standard" to judge as to how far the stained preparations resemble living structure. It is in this context that the discovery of the nucleus in a living zygote is interesting. An accurate evaluation of the effects of fixatives and stains on the living nucleus of yeast is now available (Royan, 1956 *a*, and unpublished observations).

Considerable care has to be exercised in the selection of the standard. According to Wager (1898), Nageli and Henneguy had observed the nucleus in living yeast cells with an ordinary microscope. Guilliermond's (1910) comment that the nuclei of zygotes and vegetative cells show an identity in structure in stained preparations necessitates the selection as a standard a living zygote nucleus having a nuclear membrane enclosing formed structures inside.

Because the living nucleus is visible only under particular physiological conditions in some strains of yeasts (Royan and Subramaniam, 1956; Royan, 1956 *a, b, c*; Thyagarajan and Subramaniam, 1957) the easy availability of the phase contrast microscopes did not produce any spectacular advances in yeast cytology. According to Mundkur (1954) and Muller (1956) the nucleus appears often only as a homogeneous body under the phase contrast type of illumination. If such a living structure is taken as the standard, then, the stained nucleus should appear as a 'solid' body and structural details if revealed by fixatives and stains are likely to be considered as artefacts.

The function of a good fixative is to produce a life-like preservation of the cell organelles. Since the living nucleus is taken as the standard, the fixative should preserve not only the nuclear structure but also that of the vacuole. Iodine-formaldehyde-acetic acid mixture was the fixative of choice in view of Royan's (1956 *a*) demonstration that apart from giving a life-like preservation, the nucleus itself could be followed up under the microscope during the cytological procedures like fixation, hydrolysis and staining.

The cells from the 7-day culture were centrifuged, washed well with distilled water and fixed in a freshly prepared mixture containing 9 ml. of Gram's iodine, 1 ml. of 40% formaldehyde and 2 drops of glacial acetic acid. After a stay of one hour in the fixative the cells were sedimented by centrifugation and smeared one cell thick on slides coated with Mayer's albumen. They were stored for one hour in 70% alcohol to remove the iodine and then washed in running water for 45 minutes. The slides were rinsed twice with distilled water and hydrolysed in  $\text{NHCl}$  at 60° C. for 8–10 minutes to remove the cytoplasmic basophilia (Sinoto and Yuasa, 1941; Royan, 1956 *a*).

*Staining with Hæmatoxylin.*—The hydrolysed cells were rinsed in distilled water and mordanted in 4% ferric ammonium sulphate for 16–24 hours. After a wash in running water for 30 minutes they were transferred to 0.5% well ripened hæmatoxylin for 48 hours. The smears were carefully differentiated in 2% iron alum, washed in running water for one hour, dehydrated

through ascending grades of alcohol, cleared in alcohol-xylol mixtures followed by pure xylol and mounted in canada balsam.

The structure of the zygote nucleus in iron-hæmatoxylin preparations (Photo 11) resembles that seen in the living zygotes. A well stained nuclear membrane delimits the nucleus from the cytoplasm. The formed structures visible inside the living nucleus are stained by hæmatoxylin. A comparison of Photos 7 and 11 may suggest that the stained nucleus is inferior to the living one in clarity. This is due to the technical difficulties involved in photographing nuclei having stained areas disposed at slightly differing foci.

*Staining by the Feulgen technique.*—For this purpose the hydrolysed smears rinsed well in distilled water were transferred to leuco-basic fuchsin and kept in the dark for 3 hours. They were differentiated in two changes of SO<sub>2</sub> water for 15 minutes each, lightly tinted with light green, dehydrated and mounted in canada balsam. Examination under ordinary illumination reveals a stained area (FSA, Photo 12) of variable configuration and size. The Feulgen positive area has little resemblance in structure to the body identified as the nucleus in the living cells (compare Photos 6, 7 and 8 with 12). If the nucleus was not visible in the living zygote, there is the likelihood of the Feulgen positive area being identified as the nucleus (Winge, 1935; Ganesan, 1956) in spite of differences between hæmatoxylin and Feulgen preparations. Lietz (1951) invites attention to the salient fact that the area stained by leuco-basic fuchsin forms only a portion of the nucleus. Heidenhain's hæmatoxylin is a regressive stain and one could stop differentiation at a stage to conform to the Feulgen picture (Ganesan, 1956). But the standard should be the living nucleus and not a Feulgen preparation.

Unfortunately the ordinary microscope does not reveal clearly the nuclear structure in Feulgen preparations. Examination under phase contrast becomes imperative. The structure of the nucleus is then clear. The Feulgen positive areas show varying dispositions inside the space bounded by the nuclear membrane (Photos 13 and 14).

#### DISCUSSION

A zygote is usually defined as the product of union of two gametes (Wilson, 1904). This term naturally embraces the individual originating from the product of fusion (Darlington, 1932). The buds arising from an yeast zygote constitute a population of individual cells. It would be desirable, therefore, to have a clear grasp of the connotation of the term zygote in yeasts.

Zygotes are reported to originate in yeasts in diverse ways (Phaff and Mrak, 1948). In some genera like *Schizosaccharomyces* the vegetative cells fuse in pairs to give rise to zygotes. Spore formation occurs in these zygotes (Royan, 1956 *d*). In the genus *Saccharomyces*, on the other hand, the vegetative cells give rise to asci with spores. Two spores when they fuse give rise to a spore zygote. In some cases the spores germinate directly and the resulting cells may fuse in pairs. The progeny of an isolated spore are reported to be capable of fusing with an ungerminated spore itself (Winge, 1935). It has been suggested that even the first two nuclei in a germinating spore may fuse to give rise to a diploid cell (Winge and Laustsen, 1940). During the formation of zygotes plasmogamy is presumed to be followed by karyogamy. In some cases, however, karyogamy is said to be delayed resulting in "dicaryotic" stages (Guilliermond, 1910; Renaud, 1938; Phaff and Mrak, 1948).

The zygotes illustrated (Photos 1-14) and described in this paper are formed by the fusion of pairs of spores. The nuclei of these zygotes have the structure suggested by Guilliermond. The existing reports on the nuclei of zygotes do not all relate to those formed by the fusion of spores. While Guilliermond's (1910) description is of dicaryotic stages in spore zygotes, Winge's (1935) observations relate to cell zygotes. It becomes relevant in this context to consider whether the divergences of opinion regarding the structure of the nucleus may be the result of differences in the mode of origin of the zygotes investigated by them. The similarity in the structure of the living nuclei of the zygote and bud illustrated in Photos 9 and 10 would imply that differences in the mode of origin may have no relation to the structure of the zygote nucleus.

The illustrations of the nuclei of living zygotes presented in this paper are from 7-day cultures. Naturally, these nuclei cannot be taken as representing the condition immediately on fusion of two spore nuclei. Since the nucleus of the zygote (Photo 9) retains its condition even after giving rise to the nucleus of the bud (Photo 10) it has to be presumed that the "fusion" nucleus has the same structure as the nucleus remaining in the zygote after giving rise to one or more buds. The similarity of structure of the nuclei of vegetative cells (Thyagarajan and Subramaniam, 1957) and zygotes (Photos 5, 6, 7 and 8) would reinforce such a conclusion.

#### SUMMARY

1. The zygotes of *Saccharomyces carlsbergensis* retain the shape assumed at the time of their origin by the fusion of two spores even after they have produced a few buds.

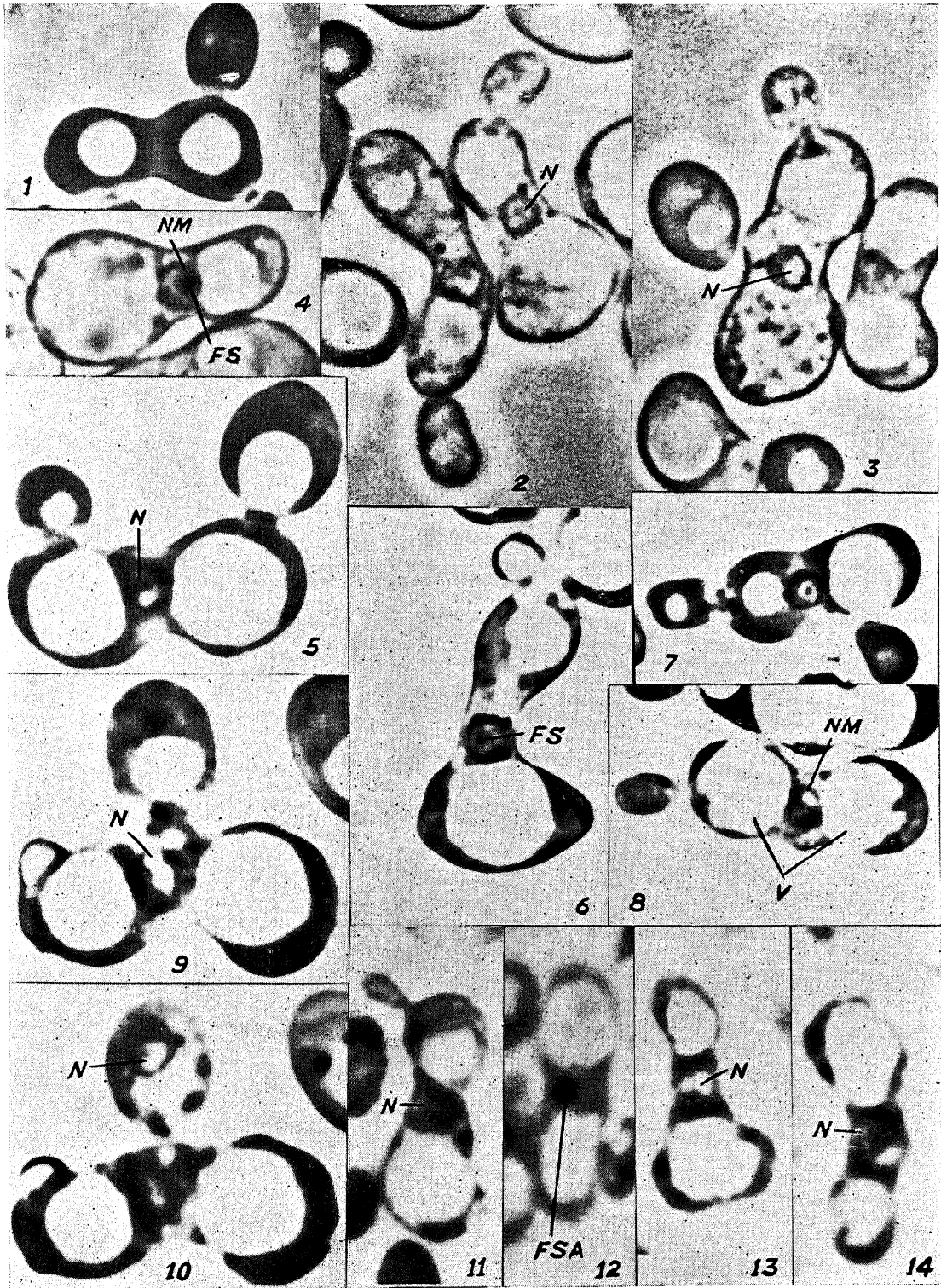
2. The nucleus is visible in the zygotes on the 7th day after the introduction of spores into fresh wort. A nuclear membrane delimits the nucleus from the cytoplasm and encloses formed structures. The structure of the nucleus is identical in a zygote and its bud.

3. Iodine-formaldehyde-acetic gives a life-like preservation of cell structures. After removal of the cytoplasmic basophilia by acid hydrolysis, staining with hæmatoxylin gives pictures of nuclear details comparable to that of the living nucleus. The Feulgen stained area is only a portion of the resting nucleus.

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#### DESCRIPTION OF PHOTOMICROGRAPHS

- PHOTO 1. A zygote from a 3-day culture showing two vacuoles. The nucleus is not visible (Phase Contrast).
- PHOTOS 2, 3 & 4. Zygotes from 7-day culture. The nuclear membrane (NM) encloses formed structures inside the nucleus. A crescentic denser area attached to the inner surface of the nuclear membrane is visible in Photo 3, while a grain is present in Photo 4 (Ordinary Illumination).
- PHOTO 5. A zygote with two attached buds of differing ages. The nuclear area is clear (Phase Contrast).
- PHOTO 6. The nucleus has the appearance of a dense ring enclosing a distinct grain (Phase Contrast).
- PHOTO 7. The nuclear periphery shows irregular thickening. Note the central grain (Phase Contrast).
- PHOTO 8. A dense sickle-shaped mass is plastered on to the inner surface of the nuclear membrane (Phase Contrast).
- PHOTOS 9 & 10. Nuclei in a zygote and its bud (Phase Contrast).
- PHOTO 11. The structure of the zygote nucleus after staining with hæmatoxylin (Ordinary Illumination).
- PHOTO 12. The area stained by leuco-basic fuchsin in hydrolysed cells (Ordinary Illumination).
- PHOTOS 13 & 14. Under phase contrast the Feulgen positive areas show varying dispositions inside the space bounded by the nuclear membrane.

Magnification,  $\times 4,200$ . N., Nucleus. NM., Nuclear Membrane. FS., Formed structures visible inside the nucleus. FSA., Feulgen stained area. V., Vacuole.