

DIFFERENTIAL FLUORESCENCE OF THE CHROMOCENTERS AND NUCLEOLAR EQUIVALENTS OF THE YEAST NUCLEUS IN ACRIDINE ORANGE

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INTRODUCTION

THE living nucleus of yeast shows formed structures within the area enclosed by the nuclear membrane (Henneguy, 1896; Royan, 1956 *a, b*, 1958 *b, c*; Thyagarajan and Subramaniam, 1957 *a, b*; Thyagarajan, 1959; Aswathanarayana and Subramaniam, 1958; Subramaniam, Royan, Thyagarajan, Aswathanarayana and Subramanyam, 1959). These organelles could be stained with hæmatoxylin (Royan, 1956 *a*, 1958 *a*; Thyagarajan and Subramaniam, 1957 *b*; Aswathanarayana and Subramaniam, 1958). Some of them were Feulgen positive. The Feulgen negative structures were suspected to be the nucleolar equivalents. Cells fixed in iodine-formaldehyde-acetic acid solution and stained with Giemsa, without any hydrolysis, showed a differential staining of these two organelles (Royan, 1958 *c*). The chromocenters were red while the nucleolar equivalents were blue.

These conclusions drawn from fixed preparations necessitated confirmation, if possible, by vital staining. If examination of material was to be limited to observations with an ordinary microscope, then, no vital stain capable of such a distinction is available. The fluorochrome, acridine orange, has been claimed (Armstrong, 1956; Smiles and Taylor, 1957; Bishop and Austin, 1957; Bishop and Smiles, 1957; Austin and Bishop, 1959) to enable a distinction of the cell organelles by their differing fluorescence. An attempt was, therefore, made to elucidate whether the intranuclear structures of yeast exhibit a differential fluorescence when stained with acridine orange.

MATERIAL AND METHODS

The earlier observations on the strain, *Saccharomyces cerevisiæ*, NRRL, Y. 567 (Royan and Subramaniam, 1956; Royan, 1956 *a, b, c*, 1958 *a, b, c*) were carried out using barley malt wort of sp. gr. 1.020 and pH 4.6-4.8. During an exploration of alternative media suitable for observations on

the nucleus in the living condition, it was discovered that a medium (MPYA) having the composition, maltose (City Chem. Corp., N.Y.) 10 gm., peptone (Difco) 1 gm., yeast extract (Difco) 0.5 gm. and asparagine (Nutritional Biochem. Corp., N.Y.) 0.2 gm. dissolved in 100 ml. of distilled water and the specific gravity of which was adjusted to 1.020 and the pH to 4.6-4.8 would serve equally well. Unlike in malt wort, the nuclei were visible only in a smaller percentage of cells from the eleventh day onwards. Photos 1 and 2 show cells with nuclei from a 6-day barley malt wort culture and from a 13-day MPYA medium respectively. There is no major difference in the structural details of the nuclei.

Though the ideal temperature for culturing the strain is 25° C. the progress of aging, after the nuclei have become visible, was rapid with a consequent granulation of the cytoplasm. This aging process was slowed down by transferring the 11-12 day old cultures to a refrigerator (5-7° C.).

The source of ultra-violet light was a Zeiss super pressure mercury lamp with a heat filter, a blue energizing filter and an orange-coloured check filter for the eye-piece. The blue filter is said to permit essentially the 4 g lines at 366, 403, 405 and 436 m μ as fluorescent energizing light to pass through. The microscope used for examination had a condenser 1.4 N.A and an objective \times 97 with an iris diaphragm. The stained preparations were photographed with a Leitz Mikro attachment giving a 1:1 magnification on Kodak Microfile and Ektachrome films.

OBSERVATIONS

Acridine orange (E. Gurr) was dissolved in pyrex distilled water. The determination of the optimal concentration of the dye was by testing a series of dilutions. When the concentration of the dye was 1 in 20,000, the staining was rapid and the cytoplasm assumed quickly a reddish orange fluorescence. It was only in dilutions above 1 in 30,000 that the staining was relatively slow. A dilution of 1 in 40,000 was preferred because the chromocenter was green while the nucleolar equivalent was orange. A concentration suitable for cells grown in MPYA medium was found to stain some of the cytoplasmic granules reddish orange in those grown in barley malt wort.

The procedure adopted was to mount a few cells in a drop of acridine orange (1:40,000) and flatten them under the coverslip thirty minutes later, by removing the extra stain with filter-paper strips. They were then sealed with paraffin wax, kept in the dark and examined periodically.

Another method was to centrifuge out the yeast cells from 1 ml. of a well-shaken 10-14 day MPYA culture and suspend them in 5 ml. of acridine orange (1:40,000) in a test-tube. After a stay of 24 hours in the dark, samples from the stain were sealed under a coverslip with paraffin wax and examined periodically. When mounted, the cells, with the exception of the dead and senescent ones, were green and exhibited no orange fluorescence. The nuclear details were clear owing to their differing intensities, the nucleolar equivalent being prominent. It was only after the lapse of 24 hours after mounting under the coverslip that a differential fluorescence of the nucleolar equivalents and chromocenters was observed.

Transfer of cells stained for 24 hours in a test-tube, to fresh growth medium without the stain, resulted in cell proliferation. The cell deposit at the bottom of the tube 24 hours after transfer, was light orange in colour. Examination revealed the persistence of the dead and senescent cells fluorescing orange. The budding cells were dull green.

The first procedure was preferred owing to the shorter time required for the differential fluorescence to appear in the cells. It should be emphasized that not all cells show a uniformity in the nature of the polychromatic fluorescence. If the cells were very few, they showed an orange halo around them on mounting which disappeared subsequently. The cytoplasm of the majority of such cells was green at the beginning. Many of them turned yellow and quickly assumed a bright orange fluorescence. Even then the chromocenters remained green.

If there were a large number of cells under the coverslip, the majority remained green with clear cellular details. Only a few showed areas fluorescing orange. The optimum number of cells to be mounted under a coverslip could be determined only by experience. Curiously enough, the nucleus visible under phase-contrast only in a small percentage of cells stained in acridine orange (1:40,000) was clear in the majority of the cells under ultra-violet illumination. The progress of staining under optimal conditions was followed repeatedly.

The following sequence of events were observed in material mounted in acridine orange (1:40,000). A few minutes after sealing, some cells orange in colour put in their appearance. These seem to be the senescent or dead cells. The others could at first be located only as green patches with clear outlines. There was a gradual increase of the green fluorescence of the cytoplasm. The nucleolar equivalent put in its appearance as a greenish white structure. It was at this stage that the vacuole became visible as an

unstained area on a dark background. The nuclear membrane then became distinct. The chromocenters were the last to appear as structures fluorescing green. The nucleolar equivalent began slowly to show an orange fluorescence whose intensity increased with time. In some cells, however, the cytoplasm was light orange, the nucleolar equivalent bright orange, the chromocenters alone remaining green. The nuclear membrane was of the same colour as the cytoplasm. The time required for the above series of changes resulting in a clear polychromatic fluorescence of the nuclear details ranged from three to twenty-four hours.

If the cells were not exposed to ultra-violet irradiation, this characteristic polychromatic fluorescence could be observed even after the lapse of five days, if the slides were kept shielded from light. Very short exposures, as happens when a slide is scanned quickly, does not produce any loss in intensity of the fluorescence of the structures. But if specific regions are exposed for periods of more than one minute there was a tendency for the loss of the orange colour in some of the cells. These became green or greenish yellow.

The loss in intensity on exposure to ultra-violet light created difficulties for photography. The Microfile film used being a slow one, the exposure times ranged between two and a half to four minutes. Even though cells fluorescing brightly were chosen, there was a loss in intensity of the orange tint on the completion of the exposure. The pictures were better on the faster Ektachrome film. Photos 3 and 4 printed from Microfile negatives illustrate the condition on staining with acridine orange. The vacuole (V) could be seen as an unstained area in Photos 3 and 4. The nucleolar equivalent (NE) appears as a characteristic crescent attached to the nuclear membrane (NM) in the illustrations (compare with Photos 1 and 2). The chromocenter (C) lies free in the nuclear matrix.

DISCUSSION

Yasaki and Sinoto (1952) could not observe the nucleus of *Saccharomyces cerevisiae* when stained with the fluorochrome, aluminium morine. Krieg (1954) claimed that he could stain the organelle, identified by Rochlina as the nucleus of the yeast cell, uniformly with acridine orange. Rustad (1958) saw the nucleus of *Schizosaccharomyces pombe* as a vacuole of low refractive index in living cells and reported that it gave an intense green, yellow-green or yellow fluorescence. He did not observe any differential fluorescence in the nucleus.

The earlier distinction of the nucleolar equivalents and chromocenters in the yeast nucleus was by their differing affinity for the components of the

Giemsa stain. The chromocenters were red while the nucleolar equivalents were blue (Royan, 1958 *c* ; Subramaniam *et al.*, 1959 ; Subramaniam, 1960). These two organelles could equally well be distinguished using acridine orange. The chromocenters fluoresced green while the nucleolar equivalents were orange.

SUMMARY

The nucleus with its limiting membrane and organelles was visible in the majority of the yeast cells stained vitally with the fluorochrome, acridine orange, at a dilution of 1 in 40,000. The intra-nuclear structures could be distinguished by their differential fluorescence. The chromocenters were green while the nucleolar equivalents were orange. The vacuole showed no fluorescence.

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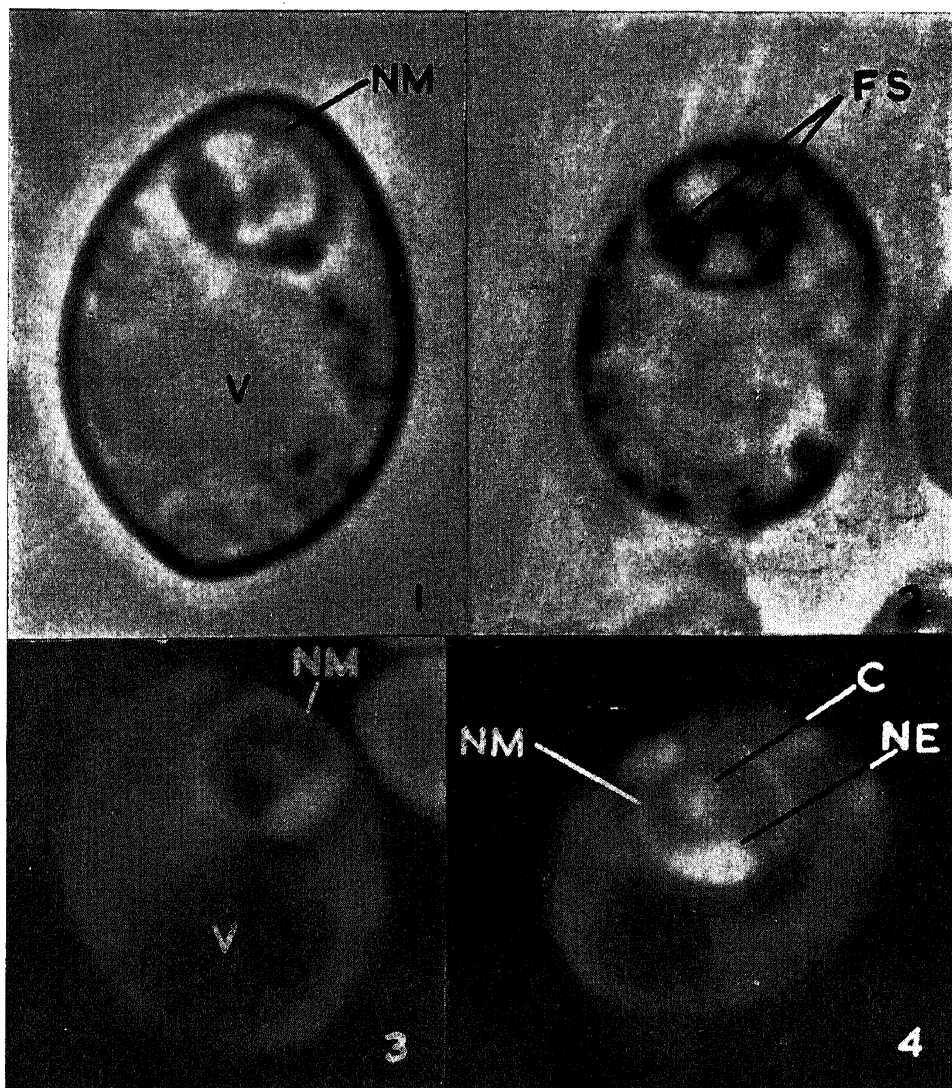
EXPLANATION OF PLATE IX

PHOTO 1. Living Cell (Barley Malt Wort), Ordinary illumination. $\times ca. 6,200$.

PHOTO 2. Living Cell (MPYA Medium), Ordinary illumination. $\times ca. 6,200$.

PHOTOS 3 and 4. Cells treated with a solution of acridine orange (1:40,000), $\times ca. 5,400$.
Note the nucleus with the distinct nuclear membrane (NM) enclosing the chromocenter (C) and the nucleolar equivalent (NE).

C, Chromocenter; FS, Intra-nuclear Structures; NE, Nucleolar Equivalent; NM, Nuclear Membrane; V, Vacuole.



PHOTOS 1-4