AN EVALUATION OF THE STANDARDS USED IN THE STUDY OF THE YEAST NUCLEUS

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INTRODUCTION

THE discovery of the nucleus in a living yeast by Nägeli dates back to 1844. It is, however, visible only in some yeasts under specified conditions (Royan and Subramaniam, 1956; Royan, 1956 *a*, *b*, *c*; Müller, 1956; Thyagarajan and Subramaniam, 1957 *a*, *b*). During the years intervening the first record of its presence in a living cell (Nägeli, 1844) and its rediscovery by Henneguy (1896; see Wager, 1898) the debate centred round the question whether yeast has a nucleus resembling that of higher organisms. The non-availability of the living nucleus as a standard necessitated dependence on fixation and staining procedures then in vogue for the study of the nucleus. The lack of agreement between the investigators (see Wager, 1898) would indicate the limitations of orthodox fixing and staining procedures when they are used as the sole standards for the study of the nucleus of yeast.

STANDARDS USED FOR EVALUATION OF RESULTS

Eisenschitz (1896) departed radically from conventional methods by attempting to stain vitally living yeast cells. He could neither see the nucleus reported by Nägeli and Henneguy nor stain it vitally. He saw only certain stained grains lying partly inside and partly outside the vacuoles. Naturally, his conclusion that the granules and vacuoles together represent the preliminary stages of a nucleus is justified by his observations.

Bouin (1898) saw a normal nucleus in fixed preparations only under certain conditions. He remarks that yeast may become plurinucleate if it is grown at a high temperature, or in media lacking some mineral constituents or even if the medium is rich in nutriments. He thought that the granules observed by some authors " represent the nucleus which has become divided by a series of divisions not followed by cellular divisions " (Wager, 1898, p. 507).

Wager (1898) saw in living cells the body identified by Nägeli as the nucleus. But the living nucleus was not ideal like that of Henneguy (1896).

It was neither delimited from the cytoplasm by a definite membrane (see however, Wager, 1898, p. 518 and Fig. 4) nor did it exhibit any internal structural differentiation. On staining, it resembled the 'nucleolus' of *Phaseolus*. It is but natural that he had reservations regarding its nuclear nature. He suggested that the body considered as the nucleus by Nägeli is only the 'nucleolus' and that the prominent vacuole is the real nucleus. Fixation and staining procedures became once again the standards for judgement. According to Wager and Peniston (1910) nuclear continuity from one vegetative generation to the next is only through the 'nucleolus'. They assigned the vacuole only the status of a vegetative nucleus. In a modern context their description would imply that yeast is bi-nucleate.

The disagreement regarding the intra- (Janssens and Leblanc, 1898; Janssens, 1903) or extra-vacuolar position of the body designated as the nucleus by Nägeli (1844) paled into insignificance with the demonstration by Caspersson and Brandt (1941) that under certain cultural conditions the cytoplasm of the yeast cell appears homogeneous because of the absence of any visible inclusions. In the context of the above discovery Subramaniam (1946, 1948, 1952) emphasised the importance of cultural conditions for an accurate evaluation of the structure and behaviour of the yeast nucleus.

The question whether the vacuole is a permanent constituent of the yeast cell can only be judged in relation to its behaviour under varying physiological conditions. It was shown recently (Aswathanarayana, 1956) that while the majority of the cells in cultures ranging in age from 5–12 days had vacuoles and grains, only a small percentage showed these inclusions when the culture was 24-hr. old (Table I). On stimulation of the cells from these cultures with fresh media the cytoplasm lost its inclusions and became homogeneous (Aswathanarayana, 1956; 1958).

Because of the difference in the morphology of the cytoplasm between the 24-hr. and 5-day cultures, Subramaniam (1946, 1948) studied them separately even though at the time of fixation they had homogeneous cytoplasm as a result of stimulation by transfer to fresh media. The living cell bereft of any vacuoles or grains was the standard for his investigations. He fixed the stimulated cells from 24-hr. cultures in Carnoy's and Bouin's fluids and stained them with iron hæmatoxylin. He described (1946) a nucleus with a nuclear membrane which resolved itself into two chromosomes during mitosis. This was in conformity with the earlier records of Badian (1937), Caspersson and Brandt (1941) and Robinow (1944).

From a parallel investigation of stimulated cells from 5-day cultures he described "endopolyploidy" (Subramaniam, 1948). The standards were

(i) cells bereft of any visible inclusions at the time of fixation and (ii) mitosis described earlier from young cultures. The description of the phenomena observed as "endopolyploidy" was rendered necessary in the context of the earlier work of Eisenschitz (1896), Bouin (1898), Wager (1898) and Wager and Peniston (1910).

The standard of Lindegren (1945) was a vacuolated cell. In Feulgen preparations Lindegren and Rafalko (1950) observed a cyclical relationship in the stainability of the structures present inside the "centrosome" (= nucleus, Nägeli) and the vacuole. The bodies stained by leuco-basic fuchsin inside the vacuole had a different tint and structure from those in the "centrosome" (see Table I, p. 171).

While Subramaniam's (1946) description of a vesicular nucleus delimited from the cytoplasm by a definite membrane has evoked little interest, his suggestion that yeast becomes "endopolyploid" under certain physiological conditions has been disputed (Winge and Roberts, 1954; Ganesan, 1956). The rationale behind the use of cells having a homogeneous cytoplasm at the time of fixation is that the structures revealed by staining are theoretically artefacts. Nissl (1910) held such a view and standardised procedures to obtain "Aequivalent-bilder" (Wolman, 1955).

The necessity for entire dependence on fixed and stained preparations gives scope for different interpretations because a particular technique becomes the standard for evaluation (Winge, 1951; Winge and Roberts, 1954; Ganesan, 1956). When cells bereft of any inclusions are fixed and stained in different ways, the structures seen in the finished preparations are artefacts. It becomes necessary, therefore, to separate (i) those which have an existence but are invisible from (ii) the real artefacts of fixation. This would be possible only if the organelles whose reactions to fixatives and stains are investigated are visible in the living cell.

A search was, therefore, made for the living nucleus in the brewery yeast BY 1, investigated earlier in this laboratory (Subramaniam, 1946).

Experimental Procedures

The observations reported recently that the nucleus is clearly visible in living cells from 96–120-hr. wort cultures of a strain of *Saccharomyces cerevisiæ* (Royan and Subramaniam, 1956; Royan, 1956 *a*, *b*, *c*) and 160-190-hr. cultures of a strain of *Saccharomyces carlsbergensis* (Thyagarajan, and Subramaniam, 1957 *a*, *b*) indicated the necessity for a systematic examination of cultures of varying ages of BY 1—a strain of *Saccharomyces cerevisiæ* —to locate the living nucleus.

Three slightly varying methods were adopted for the culture of yeast: (i) A few cells of BY 1 from 15–20-day agar slopes were transferred to tubes containing 5 ml. quantities of barley malt wort of S.G. 1·020 and pH 4·6–4·8, and allowed to grow at a room temperature of 23–25° C. Samples from these tubes were examined twice a day. (ii) When the cells from 15–20-day agar slopes had grown in wort for 16 hr., the tube was shaken well and three loops of material were transferred to tubes with 5 ml. of wort or (iii) 5 ml. of a medium containing 2% galactose, 1% peptone and 0·5% yeast extract having a pH of 4·6–4·8.

A careful survey of samples of varying ages from these three types of cultures was made with a Zeiss Phase Contrast Research Microscope. The nucleus was clearly visible only in 1–2% of the cells in wort cultures. It was seen in cells from 6–7-day cultures when the inoculum was from an old slant (method i) and in 7–8-day cultures when the inoculum was from 16-hr. wort cultures (method ii). In the GPY medium (method iii) the nucleus was visible only in 0·5–1% of the cells between the 10th and 12th day after inoculation.

Samples were mounted in a drop of the medium in which they had grown, the excess of the medium around the coverslip was removed with filter-paper and two moist filter-paper strips of equal size were placed on either side of the square coverslip to prevent the movement of the cells. They were photographed with a Leica attachment using Kodak 35 mm. ' Microfile ' film. The negatives were enlarged to varying diameters.

OBSERVATIONS

1. *Living cells.*—When visible, the nucleus appears optically denser than the cytoplasm. It lies outside the vacuole and is delimited from the cytoplasm by a definite membrane (Photos 1–8). The nuclear matrix may be homogeneous (Photos 1 and 2) or may show denser areas (Photos 3–8). In not all cells is the nuclear membrane thin. Often it is thick. The thickening may be irregular (Photo 5) or uniform (Photos 4, 6, 7 and 8).

The formed structures inside the nuclear matrix show varying dispositions. They may lie free inside the nucleus (Photos 4 and 8) or may be plastered on to the inner surface of the nuclear membrane. Their shape is granular in Photos 4 and 8, while their contour is diffuse in Photo 3. Often they have an irregular semi-lunar shape when plastered on to the nuclear membrane (Photos 5, 6 and 7). There is only a single grain in Photo 4, while there are a pair of granules inside the nucleus in Photo 8. The formed structures plastered on to the inner surface of the nuclear membrane vary in number

as well as size. A pair of unequal semi-lunar bodies occur inside the nucleus in cells in Photos 6 and 7.

Gradations of optical density under phase contrast, of the nuclear matrix itself, is illustrated in Photos 6 and 7. Since formed structures occur often on the inner surface of the nuclear periphery (Photos 5, 6 and 7), the uniformly thick nuclear membrane in Photo 4 may itself be the result of optically dense material lining the inner surface of the thin nuclear membrane.

Attention is invited to the importance of a visible nuclear membrane in judging whether the nucleus is an extra- (Royan, 1956 *a*, *b*, *c*) or intra-vacuolar (Lindegren, Williams and McClary, 1956) structure, and the separation of granules lying inside the nucleus from those outside it. The nucleus is an extra-vacuolar structure (Photos 1–8). There is a dense granule in the cytoplasm at the junction between the nucleus and the vacuole in Photo 5. In Photo 8 a pair of grains occur inside the nucleus while a vesicle lies outside the nuclear membrane.

2. *Fixed and stained preparations.*—The fact that the nucleus is visible only in a very small percentage of the cells implies that they may be exceptions. But the visibility of the nucleus in some cells enabled an evaluation of the action of fixatives and stains. Following Moeller (1892), Johansen (1940) and Nagel (1946), Royan (1956 *a*, 1958) compounded a fixative containing 45 ml. of Gram's iodine solution, 45 ml. dist. water, 12 ml. of neutral formaldehyde and 1·5 ml. of glacial acetic acid and showed that it gave a life-like preservation of the yeast nucleus. She indicated that its utility for the study of the nuclei of other yeasts could only be judged by experiments.

It was discovered that a slight modification was necessary to obtain an accurate fixation of the nucleus of the brewery yeast strain, BY 1, the subject of this paper. Gram's iodine solution had to be diluted with double its volume of distilled water before adding the requisite quantities of formaldehyde and acetic acid.

Cells from 7–8-day wort cultures were smeared one cell thick on slides coated with a thin layer of Mayer's albumin and transferred to the above fixative. After a stay of 60 minutes they were stored in 50% alcohol for periods ranging from 2–16 hr. to remove the iodine. The smears were then washed in running water for 45–60 minutes.

The disagreements regarding the identification of a particular organelle of the yeast cell as its nucleus (see Wager, 1898) could be traced to the intense cytoplasmic basophilia. It is this which makes yeast refractory to the usual stains. The cytoplasmic basophilia could easily be removed by hydrolysing

the smears with N HCl at 60° C. for 7–10 minutes (Sinoto and Yuasa, 1941; Kurnick, 1955; Royan, 1956 *a*).

(*a*) *Staining with hæmatoxylin.*—The hydrolysed cells after a thorough wash in distilled as well as tap water were mordanted in 4% ferric ammonium sulphate for 12–24 hours, washed for 20 minutes under the tap and transferred to well ripened hæmatoxylin for 24–48 hours. Destaining with 2% iron alum was carefully controlled by observation under an oil immersion objective. The smears were then washed in water for 30 minutes, exposed for a few seconds to ammonia vapour, washed again for 10 minutes, carefully dehydrated in ascending grades of alcohol, cleared in alcohol-xylol mixtures followed by pure xylol and mounted in canada balsam.

A few of the variations in the structure of the nucleus observed in hæmatoxylin preparations are illustrated in Photos 9–12. It will be seen that the vacuole shows no stained inclusions. The extra-vacuolar nucleus may appear as a solid structure showing no internal differentiation (Photo 9). Since occasionally the nucleus in a living cell appears as a homogeneous body showing no structural differentiation, the 'solid' nucleus in Photo 9 has to be presumed to have a reality. The stained area in Photo 10 represents the major portion of the nuclear matrix. The nuclear membrane is clearly seen bounding the unstained portion of the nucleus. Compared to Photo 10, a greater region of the nuclear matrix is unstained in the cell illustrated in Photo 11. The lack of uniformity in the thickness of the nuclear membrane in the above cell is reminiscent of the condition in some living cells (Photo 5). Photo 12 is interesting in that the staining is limited to one half of the nucleus. The unstained half enclosed by the nuclear membrane contains a stained granule.

(*b*) *Staining by the Feulgen technique.*—The hydrolysed cells rinsed in several changes of distilled water were kept in leuco-basic fuchsin for periods ranging from 90–120 minutes. They were then differentiated in two changes of SO_2 water for 15 minutes each, counterstained with light green, dehydrated and mounted in canada balsam.

Examination under ordinary illumination reveals that the vacuole is bereft of any organelle stained by leuco-basic fuchsin. The nuclear boundary is barely visible and only a small region inside the nucleus is stained by leuco-basic fuchsin. This stained area may be located in the centre of the nucleus (Photo 13) or may lie at the inner periphery of the nucleus (Photo 14). Under ordinary illumination the nuclear membrane is not clear in cells stained by the Feulgen technique. But it stands out clearly when the same preparations are examined under the Phase Contrast Microscope. The area stained by

leuco-basic fuchsin varies in size as well as disposition. In Photo 15 it is spherical, while in Photo 16 it is semi-lunar. These photos emphasize that (*a*) examination of Feulgen preparations under phase contrast is imperative to get a correct idea of the nuclear structure and that (*b*) the area stained by leuco-basic fuchsin forms only a portion of the nucleus.

Discussion

The earlier investigation of Subramaniam (1946) suggested that the *resting nucleus* of this strain of yeast (BY 1) is vesicular having a nuclear membrane enclosing structures of variable size and disposition stainable by hæmatoxylin. The living cell with no visible inclusions at the time of fixation being the basis for his observations, there was no possibility of determining whether the structure of the invisible living nucleus was the same as that revealed by hæmatoxylin in cells fixed in Carnoy or Bouin, or whether the fixatives had produced real artefacts.

Sinoto and Yuasa (1941) remark: " In either the living cells or in fixed unstained cells the nucleus showed a homogeneous structure and was refringent, but was discerned with difficulty from other granular bodies " (p. 465). In cells fixed in Flemming and bleached with H_2O_2 they report the existence of a karyosome surrounded by a hyaline zone and enclosed by a nuclear membrane.

Subramaniam's (1946, p. 145) Figs. 10, 11 and 12 are reminiscent of Figs. 3 *a*, *b* and *c* presented by Sinoto and Yuasa (1941, p. 466). The difficulty experienced by the above authors in distinguishing the nucleus from other cytoplasmic granules was avoided by Subramaniam by fixing cells having a homogeneous cytoplasm. The structural details of the nucleus reported by Sinoto and Yuasa from fixed and stained preparations were not visible in the living condition and hence their conclusions are based purely on stained preparations. They remark: " By the Feulgen nucleal staining method the nuclei stain faintly reddish violet and sometimes the small granules around it show a similar colour " (p. 467). It would appear that leuco-basic fuchsin stains not only the nucleus but also some cytoplasmic granules.

The nucleus of yeast according to Lietz (1951) is an extra-vacuolar structure delimited from the cytoplasm by a definite membrane. Unlike Sinoto and Yuasa (1941), Lietz found that the Feulgen technique stained only a portion of the nucleus.

Mundkur (1954) emphasizes the limitations of chemical fixatives and uses frozen dried cells for his investigations. His opinion that freeze drying is superior to chemical fixatives appears to be based on a comparison of the

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Mundkur (1954) emphasizes the limitations of chemical fixatives and uses frozen dried cells for his investigations. His opinion that freeze drying is superior to chemical fixatives appears to be based on a comparison of the

effects of these two types of fixation on living cells. He records: " Phase microscopy of living, unstained yeast shows no difference in refractivity at different points within the nucleus.......... With the ultra-violet microscope the nucleus appears as a uniformly dark spherical region in each cell " (p. 524). His conclusion that no chromosome-like structures are detectable at any stage in the cell is not surprising, since his attempt was to conform the fixed preparations to that of the living nucleus.

The use of the living yeast nucleus as a basis for a study of its real structure dates back to 1886. According to Wager (1898, p. 506) Henneguy saw in living as well as stained preparations of a red yeast " a nucleus surrounded by a nuclear membrane and possessing a nucleolus". The contradictions between Henneguy's and Mundkur's contributions on the structure of the resting nucleus are reconcilable in the context of the differences even in their observations on the living nucleus. Great care has, therefore, to be exercised in the selection of a standard. The living nucleus chosen for study should show structural differentiation.

The above analysis reveals the serious limitations imposed by the invisibility of the nucleus in the living yeast. The differing observations could be reconciled only when the living nucleus is used as a standard for investigations (Royan and Subramaniam, 1956; Royan, 1956 *a*, *b*, *c*; Thyagarajan and Subramaniam, 1957 *a*, *b*).

The micrographs of living (Photos 1–8) and stained preparations (Photos 9–16) presented in this paper confirms Subramaniam's (1946) earlier work that the resting nucleus of BY 1 is a vesicle with a nuclear membrane enclosing formed structures inside. A comparison of Photos 13 and 14 with Photos 15 and 16 would show that Feulgen technique stains only a portion of the nucleus and cannot be taken as the sole guide in evaluating the structure of the resting nucleus as has been done by Winge (1935) and Ganesan (1956).

Basing his observations on the brewery yeast, BY 1, Ganesan (1956) concludes: " A study of the photographs of the fermenting culture given in this paper show the similar nature of the stained nuclear bodies with all stains irrespective of the fixatives used " (p. 132). A comparison of Photos 1–8 of the living nucleus of BY 1 presented in this paper with those of cells stained with hæmatoxylin (Photos 9–12) and Feulgen (Photos 13–16) would show that the above statement of Ganesan has little experimental justification.

There is a remarkable similarity in the variations observed in the structure of the living vegetative nucleus of BY 1 (Photos 1–8 of this paper), *Saccharomyces cerevisiæ* (Photos 15–33 of Royan, 1956 *b*) and *Saccharo-*

myces carlsbergensis (Photos 1 *a–d* of Thyagarajan and Subramaniam, 1957 *a*). What is more, the structure of the living vegetative nucleus is in no way different from that of the zygote (Photos 1–10 of Thyagarajan and Subramaniam, 1957 *b*).

Summary

1. In the brewery yeast BY 1, the nucleus is visible in 1–2% of the cells from 6–8-day cultures in barley malt wort and 0·5–1% of the cells from 10–12-day cultures in Galactose Peptone Yeast extract medium.

2. The nucleus is extra-vacuolar and is delimited from the cytoplasm as well as the vacuole by a definite nuclear membrane.

3. Under phase contrast, the nuclear matrix shows gradations in optical density.

4. Using the living nucleus as the standard, the variations in its structure are illustrated from iron hæmatoxylin and Feulgen stained preparations of hydrolysed cells.

5. The vacuole shows no stainable inclusions and leuco-basic fuchsin stains only limited regions of the resting nucleus.

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DISCRIPTION OF PHOTOMICROGRAPHS

PLATE XXIII

(a) Living Cells

PHOTOS 1–8. Illustrate the variations in the structure of the extra-vacuolar living nucleus as observed under the phase contrast microscope, ×ca. 3,100.

PHOTO 1. A cell from 12-day culture in GPY medium. Note the well-defined outline of the nucleus.

PHOTOS 2–8. Cells from 7–8-day barley malt wort cultures.

PHOTO 3. The nuclear matrix shows denser areas.

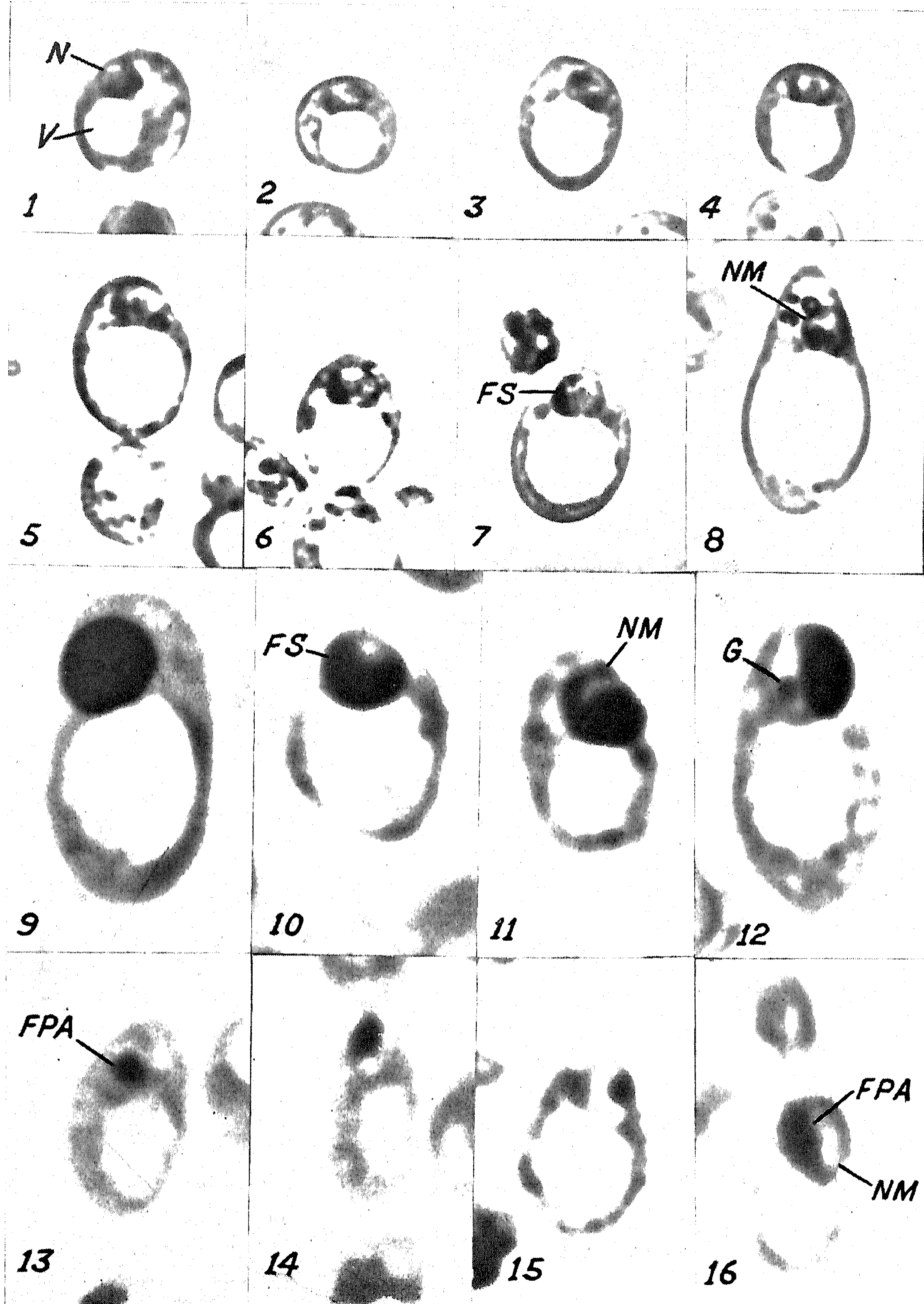
PHOTOS 4–8. The distinct nuclear membrane encloses the formed structures inside the nucleus.

PHOTO 4. A single grain is present in the middle of the nuclear matrix.

PHOTO 5. The nuclear membrane enables the separation of a granule lying inside it from that lying outside.

PHOTOS 6–7. Irregular semi-lunar structures occur plastered on to the inner surface of the nuclear membrane.

PHOTO 8. There are a pair of formed bodies inside the nucleus. A vesicular cytoplasmic structure could be seen in close contact with the nuclear membrane.



FIGS. 1–16.

(b) Fixed and Stained Preparations

PHOTOS 9–16. Cells from 8-day wort culture fixed in iodine-formal-acetic acid mixture for 60 min. and hydrolysed in N HCl at 60° C. for 8 min. before staining.

PHOTOS 9–12. Cell stained with Heidenhain's hæmatoxylin. (Ordinary illumination), ×ca. 8,700.

PHOTO 9. The nucleus is stained homogeneously.

PHOTOS 10–12 Note the nuclear membrane and the variations exhibited by the structures insid the nucleus. Unstained regions differing in area could be observed inside the nucleus.

The nuclear membrane is not uniform in thickness in Photo 11. The unstained half of the nucleus contains a well formed grain in Photo 12.

PHOTOS 13–16. Cells stained with leuco-basic fuchsin, ×ca. 6,200.

PHOTOS 13–14. The stained area occurs inside the unstained nuclear matrix. (Ordinary illumination.)

PHOTOS 15–16. Under phase contrast the nuclear membrane is seen to enclose the Feulgen positive areas.

FPA., Feulgen positive area; *FS*, Formed structure; *N*, Nucleus; *NM*, Nuclear membrane; *V*, Vacuole.