THE NUCLEAR CYTOLOGY OF THE VEGETATIVE DIPLOPHASE OF *SACCHAROMYCES CEREVISIAE*¹

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Since Schleiden in 1849 stated that he considered the yeast cell to contain a nucleus, there has been quite a controversy concerning, first, whether the yeast cell actually contains a nucleus and, secondly, what body or bodies within the cell constitute the nucleus. The reader is referred to the extensive review articles and books by Guillermond (1920), Henrici (1941), Lindegren (1945, 1949), and Lindegren and Lindegren (1946) for a summary of the pertinent literature on these subjects. In the present article only a few of the papers immediately pertaining to the problem will be touched upon.

As late as December, 1949, two opposing concepts of what constitutes the nucleus of the yeast cell were being proposed. The purpose of the present paper is to present a series of photographs representing the stages in the vegetative division of the yeast nucleus and to indicate in a brief historical review how the evidence presented here differs from the concept and data presented by Lindegren. Inasmuch as the author has been invited to present a detailed review of this subject in the near future, a complete statement of the historical and chemical aspects of the problem will be left until that time.

In a brief review of the problem four concepts of nuclear activity in the yeast must be considered. In a succession of papers covering a period of over thirty years Guillermond (1920) and his students (Renaud, 1938) presented evidence for the presence of a nucleus in the yeast cell. They further interpreted their studies as indicating that this nucleus underwent a division they considered to be amitotic. Subsequently, Guillermond (1917) conceded the probability that in *Schizosaccharomyces octosporus*, at least, the nuclear division in the ascus was probably a true mitosis, and that a mitotic division probably occurred. He stated that the chromosomes were too small to be clearly defined or to be counted. Guillermond for the most part utilized the iron alum hemotoxylin technique, which produces a blurred and indistinct picture. In 1938 Renaud, one of Guillermond's students, demonstrated the presence of a minute centrosome associated with the nucleus and showed that the vegetative division was a truly mitotic one. He too could not count the chromosomes. In 1947 Ranganathan and Subramanian confirmed the presence of minute centrosomes associated with the yeast nucleus and further substantiated the occurrence of a true mitosis in the nuclei of these organisms. The concept of the yeast nucleus and its activity, as presented by the authors already cited, is further supported by the earlier

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work of Dangeard (1893), Swellengrebel (1905), Fuhrmann (1905-1906), and Kater (1927), and is essentially the same as that to be presented in this paper (DeLamater, 1949).

A different concept of the cytology of the yeast nucleus was presented by Badian in 1937. This author, following his extensive studies on the nuclear cytology of bacteria, presented evidence that indicates that his "nucleus" is identical with that studied by Guillermond and others. He considered, however, that there is a small nuclear vesicle, and that the chromatinic mass lies at one side of this body. This chromatinic mass consists of two chromosomes which divide longitudinally in the vegetative division. This is essentially the same picture which Badian has described for bacterial nuclei, but at the present time it is not supported either by the work of Guillermond or the present author. Further details of Badian's work need not be presented here.

The third concept of the yeast nucleus was first presented by Wager in 1898 and was subsequently extended by Wager and Peniston in 1910. This work is important because it is assumed by Lindegren (1945, 1949) and his associate (1946) to be essentially correct in detail and he uses it as the basis of his concept of what constitutes the yeast nucleus and its included chromosomes.

Wager considered the yeast nucleus to consist of and include the central vacuole of the cell, and he described a delicate chromatin network within this vacuole. He considered the heavily staining body at the side of the vacuole to be the nucleolus and thought that each of the chromosomal threads in the vacuole was attached to this "nucleolus."

In the work of Guillermond (1917, 1920) and Badian (1937), on the contrary, the central vacuole of the cell is considered to be a separate entity, and the body that Wager calls the "nucleolus" is in fact the nucleus of Guillermond and Badian. Wager thought that the "nuclear vacuole" was capable of expansion and contraction and that it could even become fragmented. He considered the division amitotic. The nucleolus (the nucleus of Guillermond and others) he described as homogeneous, but often with a denser mass at one side. He never observed spindles and he was unable to count chromosomes. Nor was he certain that the granules present within the vacuole were chromosomes, but described them as "volutin granules." During the vegetative division he observed a portion of the vacuole to be pinched off into the base of the bud, and the nucleolus was observed to divide in essentially the same manner as that described by Guillermond for the division of the body he considered the nucleus.

The views presented by Wager and by Wager and Peniston have been extended in recent years by Lindegren (1945, 1949) and associate (1946) to form the fourth concept of the yeast nucleus. Lindegren presents evidence for the yeast nucleus as consisting of the central vacuole of the cell, within which are granules or bodies which he considers to be the chromosomes. The darkly staining mass at the side of this vacuole, lying within the cytoplasm of the cell, he calls the "centrosome." By using techniques that will be described in more detail presently, he presents photographs and drawings which he believes indicate that there are six pairs of chromosomes in the vegetative diplophase. In

preparation for division these become aggregated into two dense bars, which subsequently divide longitudinally to form four dense bars. All of these bodies, as they undergo these activities, lie within the central vacuole ("nuclear vesicle"). This vacuole then pushes a budlike process into the base of the bud; the four dense bodies then disintegrate into their component chromosomes, which at this stage are very minute, and one cluster migrates into the base of the daughter cell. By use of Rafalko's (1946) modification of the Feulgen technique, Lindegren claims to have obtained evidence for the presence of DNA in these intravacuolar bodies that he considers to be chromosomes.

The dense mass that lies at the side of the vacuole—the nucleolus of Wager, the nucleus of Guillermond, which has been found by Margolena (1932), Rochlin (1933), Imšenecki (1936), Wingë and Lausten (1937), Wingë and Roberts (1948), and ourselves to be Feulgen-positive—he considers to be the centriole, and suggests that this may be equivalent in yeast to the heterochromatin in higher organisms. These centriolar bodies cannot be, or be composed of, chromosomes, he thinks, because there are only two component particles and he claims that his genetical evidence requires a minimum of four chromosomes.

Lindegren's technique for the demonstration of what he considers to be chromosomes uses methylene blue, or toluidine blue, which is applied to unfixed cells. It is applied in a mixture of formalin and acetic acid and is considered to be specific for metachromatin (volutin). This staining procedure, according to Lindegren, disintegrates or aggregates the yeast chromosomes, causing them to appear either as numerous small granules or as one large granule. Rafalko claimed that by means of his modification of the Feulgen reaction it is possible to get a positive stain of these same granules. Lindegren quotes Wiame as having demonstrated that these bodies probably consist of a "metaphosphate," which he uses as further evidence for the occurrence of what he considers "volutin chromosomes."

From a strictly chemical point of view the work of Margolena (1932), Rochlin (1933), Imšenecki (1936), Wingë and Lausten (1937), Wingë and Roberts (1948), DeLamater (1948*a,b,c*, 1949), Barger and DeLamater (1948), and DeLamater, Mescon, and Barger (1950) indicates that with proper adjustment of the Feulgen technique no stain of intracytoplasmic or intravacuolar granules is obtained, and that only the dense body at the side of the vacuole can be stained by this technique. Proper hydrolysis of the cells in hydrochloric acid is necessary. Two things seem likely, either that Rafalko obtained the so-called plasmal reaction of Feulgen or that he was not washing his preparations sufficiently in sulfurous acid following their exposure to the Schiff reagent and, therefore, was getting a nonspecific reaction in incompletely hydrolyzed ribonucleic acid granules.

The methylene blue and toluidine blue techniques, promulgated by Lindegren, are nonspecific and depend upon the characteristics of all basic dyes. In summary it may be said of the cytochemical work (Delaporte and Ronkhelman, 1937) which has been done on the yeast cell that the evidence indicates that the dense body at the side of the vacuole is the only Feulgen-positive body in

the yeast cell and that the granules present in the vacuole are ribosenucleic acid particles which take up nonspecific basic dyes in a nonspecific manner. At present, Lindegren has not proved his premise that such things as "volutin chromosomes" exist in this or any other organism.

The evidence to be presented in this paper substantiates and extends the work done by Guillermond, Renaud, Ranganathan and Subramanian, Wingë, and others, and indicates that the true nucleus of yeast is the densely staining body at the side of the vacuole. Further, all the activities to be described here have been observed to occur within this body. Henrici (1941) has said that the size of the nuclei and chromosomes of yeast probably precludes the possibility of accurate chromosomal counts because the limits of resolution of current optical systems would of necessity be pressed beyond their capacity. The present author is not convinced that this need be the case and feels that, by the proper handling of techniques that are of both a selective or specific chemical nature, considerable detail about the activity of the yeast nucleus can be obtained and that probably a definitive chromosomal count can eventually be made.

The work presented in this paper is in direct opposition to the concept propounded by Lindegren.

MATERIALS AND METHODS

The yeast strains used in these studies were obtained from Dr. Speigelman. For purposes of the present study they were grown on glucose nutrient agar prior to their fixation. Only the vegetative diplophase was studied in this work. Numerous preparations of the vegetative haplophase have also been made, and the results of these will be presented elsewhere.

Preparations were made according to the methods described by DeLamater (1948 *a,b,c*), and extensively applied to various pathogenic fungi. The method is essentially as follows: Organisms are grown in test tubes and at various periods of incubation are fixed *in situ* with Schaudinn's fixative, which consists of two parts saturated bichloride of mercury, one part absolute alcohol, with about 2 per cent acetic acid added. The fixative is heated to 60 C and poured on the cultures while hot. The cultures are agitated by means of a platinum loop and freed from the surface of the agar. They are then poured into 15-ml conical centrifuge tubes and allowed to be fixed for a period of not less than two hours. Subsequent operations are all performed in these centrifuge tubes by means of an International clinical centrifuge. The cells are spun down, the supernatant is decanted, and the cells are resuspended in distilled water. They are then recentrifuged, 1 N HCl heated to 60 C is added, and the test tubes are shaken to resuspend the cells. The tubes are then placed in a hot water bath at 60 C. Acid hydrolysis at this temperature was found to be optimal at between 8 and 10 minutes, at which time the tubes are removed from the hot water bath and placed in an acetone dry-ice mush in order to stop hydrolysis by rapid cooling. They are then immediately centrifuged, and the acid supernatant is decanted. A solution of 1 per cent formaldehyde is then applied to the cells and allowed to act for 2 minutes, after which time the cells are again centrifuged and the super-

natant removed. In a similar manner the cells are washed briefly in distilled water. A solution of 0.25 per cent basic fuchsin in $\frac{1}{25}$ N HCl is then added and allowed to stain for a period of 4 minutes. The cells are again centrifuged and the stain replaced by distilled water. They are then progressively dehydrated in 10, 20, and 30 per cent alcohol, through two changes of acetone, and then into xylene. Excess xylene is removed and the mounting medium is dropped into the test tube, drop by drop, and the cells are suspended in this. Mounts are made by placing a drop of the cells suspended in the mounting medium on a clean glass slide; this is then covered with a cover slip. Such preparations are permanent.

Feulgen preparations are made in essentially the same manner. Following fixation the cells are hydrolyzed, washed, and stained with Schiff's reagent. They are then rinsed in H_2SO_3 , and the recorded procedure is used to dehydrate and mount. The results are similar to those obtained by the formaldehyde-mordanted, basic fuchsin technique described. From a cytologic point of view, however, even the best Feulgen reaction does not give a sufficiently dense stain for excellent photographic results.

It was found that the Feulgen reaction itself could be augmented by exposing the cells to 1 per cent formaldehyde prior to their exposure to the Schiff reagent. This procedure undoubtedly produces a more nonspecific reaction as the walls of the cells stain under these conditions, but the nuclei become intensely stained. Provided the ribosenucleic acid has been removed from the cytoplasm by adequate acid hydrolysis, no staining of the cytoplasm or the vacuole and its contents occurs with the Feulgen reaction. Minimal diffuse staining of the cytoplasm occurs with the aldehyde-mordanted basic fuchsin stain or with the aldehyde-mordanted Schiff reaction.

Application of the Robinow technique, in which methylene blue (Giemsa) is used following acid hydrolysis, gives the same cytologic picture as that to be described.

The photographs for the most part were taken at a primary magnification of $1,700\times$. This magnification was doubled in printing.

OBSERVATIONS

As a preliminary to the description of the observations that follow, it is perhaps justifiable to emphasize that the sequence of events itemized here has been observed to occur only within the dark-staining body that lies outside the cell vacuole, and that no contact or continuity of any structure within the vacuole with this body has been observed. The structure to be described, therefore, represents the same structure that Lindegren considers to be the "centrosome" of his nucleus and that Wager believes to be the "nucleolus" of his nucleus. The author feels that it is the body considered to be the nucleus of the yeast cell by Guillermond, Renaud, Ranganathan, Wingé, and others.

It should also be emphasized that the process of hydrolysis clears the cytoplasm to such a degree that it is frequently difficult to visualize the limits of the vacuole. Where this vacuole is clearly visible in certain of the preparations its presence and individuality will be pointed out.

Figure 1

No. 1. Early resting or interphase nuclei in mother and daughter cells. Chromatin dense and clumped. $\times 3,400$.

No. 2. Later interphase nuclei showing chromatinic material becoming less dense and spreading out on delicate threads. $\times 3,400$.

No. 3. Still later development of nuclei into interphase or resting stage with further dispersion of chromatin. $\times 3,400$.

In figure 1, no. 1, is shown a yeast cell with its daughter bud. Within each of these is a round, dense chromatinic mass. This is interpreted as an early interphase nucleus which just precedes the stage of enlargement and elongation of the chromosomes. Number 2 shows a cluster of such cells in which the component chromatinic bodies are becoming more spread out and less dense. In the central upper cell in the photograph differences in density within the nucleus can be seen, and in the center of the nucleus is a single dense granule, thought to be the true nucleolus. In the cell to the right in this photograph the enlarging nucleus is ring-shaped, at the left of which is a dense granule also considered to be a nucleolus. In no. 3, the process of nuclear enlargement has progressed, and in the second cell from the top the nucleus is seen to contain two or three delicate threads, which appear to center on, or at least to be attached to, a dense granule, again thought to be the nucleolus. In nos. 4 and 5 much larger nuclei are shown in which the elongated chromosomal threads can be made out with greater clarity. Such entangled chromosomal threads are particularly obvious in the nucleus of the cell in no. 6. In nos. 7 and 8 are rather large interphase nuclei in which dense granules thought to be nucleoli are to be seen. The nucleolus lies in the center of the nucleus in the left-hand cell of no. 7, to the left of the rather larger nucleus in the right-hand cell of no. 7, and to the left of the nucleus shown in the cell in no. 8. In no. 9 are seen two large interphase nuclei. The upper one is within a cell that is developing a daughter bud, apparently in anticipation of a division for which the nucleus is not yet prepared. This anticipation of a nuclear division by the formation of the primordium of the daughter cell well in advance of nuclear reorganization and division is commonly observed. It is also to be seen in no. 10 in both of the centrally placed cells and in the cell in no. 11. The nucleus in the cell in no. 11 clearly shows the filamentous character of the chromosomes in the interphase nucleus, and the nucleus in no. 12 indicates that they lie in juxtaposition to the nuclear membrane.

As the nucleus prepares for the division to follow, the chromosomal threads tend to contract and come to lie at one side of the nucleus, and occasionally, as seen in figure 2, no. 1, delicate tips of the chromosomes may be seen contracting into this mass. More commonly, as seen in nos. 2 to 6, the chromosomes appear to contract into dense balls and condense into a dense cluster,

No. 4. Interphase nucleus with chromosomes in filaments lying against nuclear membrane. Nucleus is a spherical vesicle. $\times 3,400$.

No. 5. Interphase nucleus showing chromosomes as delicate threads within nuclear vesicle. $\times 3,400$.

No. 6. Similar interphase nucleus with chromosomes as threads within nuclear vesicle. $\times 3,400$.

No. 7. Interphase nucleus. Chromosomes in threads. Dense beads or granules apparent on them. In daughter bud to left nucleolus obvious in nucleus. $\times 3,400$.

No. 8. Interphase nucleus in which chromosomal threads are visible with nucleolus apparent at left. $\times 3,400$.

Nos. 9-12. Further pictures of interphase nuclei showing bud cells originating from parent cells even while nuclei are in interphase. Origin of bud has no relationship to axis of cell or position of nucleus. $\times 3,400$.

Figure 2

No. 1. Early prophase showing contraction of chromosomes to side of nucleus. Chromatin mass irregular. $\times 3,400$.

No. 2. Early prophase showing contraction of chromosomal threads into dense granules. $\times 3,400$.

Nos. 3-7. Further concentration of chromosomes into dense granules. Four such masses are apparent. Number 6 shows cell vacuole to be distinct from nucleus. $\times 3,400$.

No. 8. Prophase showing 4 contracting chromosomal bodies, 2 long and 2 round, all of which are attached to central body thought to be the nucleolus. $\times 3,400$.

Nos. 9-11. Dense contracted prophase nucleus, distinct from cell vacuole, lying near base of bud. $\times 3,400$.

No. 12. Dense prophase showing centriole. These stages are common and strongly suggest a parachute. $\times 3,400$.

No. 13. Similar prophase with centriole. $\times 3,400$.

No. 14. Later prophase showing division and separation of centriole to form polar bodies. $\times 3,400$.

Nos. 15-19. Metaphase stages showing true spindles with clumped mass of chromosomes lying between polar bodies. Axis of spindle has no obvious relationship to origin of daughter cell. $\times 3,400$.

Figure 3

No. 1. Anaphase stage. Polar bodies are separating and spindle is becoming elongated. Chromosomal mass is pulling out. $\times 3,400$.

No. 2. Later anaphase or early telophase stage. Chromosomal mass much elongated. Polar bodies no longer apparent. $\times 3,400$.

Nos. 3-5. Telophase stages showing further elongation and pulling out of chromosomes. These stages are interpreted as indicating that there is a second period of chromosomal elongation during this stage when the chromosomes are actively stretched out as they are pulled apart. $\times 3,400$.

No. 7. Late telophase showing chromosomal mass well stretched out and bending to enter base of bud. $\times 3,400$.

No. 8. Telophase stage in which the chromosomes appear knotted at center, suggesting that as they divide and pull apart longitudinally the central segments have not yet separated and the knotted part represents the portions of the dividing chromosomes which are still double. $\times 4,000$.

No. 9. Telophase showing double texture of dividing mass and knotted central portion. $\times 3,400$.

Nos. 10, 11. Anaphase-telophase stages in which division of chromosomes appears to be more irregular. $\times 3,400$.

No. 12. Late telophase as daughter nuclei separate. Daughter nucleus has yet to migrate into daughter cell. $\times 3,400$.

No. 13. Separated reorganizing nuclei. Daughter nucleus migrating into base of bud cell. $\times 3,400$.

No. 14. Dense reorganizing interphase nuclei. Polar body of nucleus in present cell still apparent. $\times 3,400$.

as shown in no. 7. Rarely the contracting chromosomes are seen as shown in no. 8. Here the chromosomes are seen as dense rods and delicate ball-like structures apparently attached to a central granule, construed as the nucleolus. In no. 6 and in no. 9 the dense chromosomal mass is seen to be distinctly separated from the central vacuole of the cell. In no. 9 this dense chromosomal mass lies at the base of the bud, between the base of the bud and the vacuole of the cell. The same is true of the nucleus shown in no. 10.

In confirmation of the observations of Renaud and of Ranganathan and Subramanian can be seen a delicate centriole forming opposite the chromosomal mass in figure 2, nos. 11, 12, and 13. This minute granule enlarges considerably, as shown in no. 13, and subsequently divides, and the two granules separate, as seen in no. 14. These granules migrate to opposite sides of the chromosomal mass to form a characteristic metaphase spindle, as shown in nos. 15 to 19. The axis of the spindle may be at an angle to, perpendicular to, or directly parallel to the axis of the bud. Apparently there is no relationship between the origin of the bud and the axis of the nuclear division.

As the division proceeds, as seen in figure 3, nos. 1 to 12, the polar bodies formed by the divided centrosomes move apart and the chromosomal mass becomes fuzzy and less densely stained. This is shown in no. 1. As the chromosomal mass divides, it is stretched out into a progressively longer pattern and tends to simulate what is observed when taffy is pulled. It is surmised on the basis of the observations made that the chromosomes are actively pulled apart during the process of division and become stretched, so that there is a second period of chromosomal elongation during the nuclear division in this group of organisms. The pictures presented are in essence identical to those given by Guillermond and others at this stage of the nuclear division. Individual chromosomes cannot be made out.

In further confirmation of the lack of correlation between the point of origination of the bud cells and the axis of the dividing nucleus, the dividing chromosomal mass can be observed in figure 3, nos. 5 and 7, to be twisting and contorting in such a manner as to suggest that it is being pulled around corners as the division proceeds, and the daughter nucleus is being drawn into the bud. Occasionally a moderate degree of definition of the chromosomal masses during these anaphase-telophase stages can be observed, suggesting that the chromosomes split longitudinally and that the adherent ends stick together and separate at the last moment in the central part of the attenuated chromosomal mass. The nodules or bumps that are present in the division stages in nos. 8 and 9 suggest this adherence of the tips of the chromosomes in these last stages. Number 9 is of particular interest because it suggests the type of figures that have been presented by Badian; on the basis of these he formulates his interpretation. The anaphase-telophase division figures presented in nos. 10 and 11 show irregularities in the late division stages because individual granules can be made out within the chromatinic mass even at this stage. A late telophase stage is shown in no. 12, in which the daughter nucleus has yet to migrate into the daughter cell. In no. 13 are shown two divided nuclei, one of which is in the

process of migration into the base of the bud. Number 14 shows a similar stage in which the polar body of the parent nucleus apparently persists even at this stage.

COMMENT

This series of photographs, demonstrating the sequence of events in the nuclear division of the vegetative diplophase of *Saccharomyces cerevisiae*, indicates that the nucleus is actually the chromatinic body lying at the side of the cell, which Lindegren considers to be the centrosome. All the stages of division that are here described have been demonstrated to occur within this body, and at no time in preparations made in this study have intracytoplasmic or intravacuolar granules, which Lindegren considers to be chromosomes, been observed. It is felt on the basis of the type of nuclear figure not infrequently observed and shown in figure 2, no. 8, that the chromosome number in the diplophase of this strain of this organism is probably four, although further study may increase this number as techniques are improved.

The presence and activity of the centriole, as described by Renaud and subsequently by Ranganathan and Subramanian is confirmed, and this is seen to be quite different and distinct from the "centrosome" described by Lindegren.

It is perhaps justifiable to point out that the organization of the yeast nucleus, as presented here, corresponds to what has been described and accepted for other fungi.

Further studies into the cytochemistry of the yeast cell are in progress, and a better definition of the organelles of this organism should be made possible by application of both new and established cytochemical methods.

SUMMARY

The nuclear activities of the vegetative diplophase of *Saccharomyces cerevisiae* have been described, and photographs substantiating the description are presented.

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