MITOSIS IN VEGETATIVE NUCLEI OF NEUROSPORA CRASSA[1]

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FOR a number of years *Neurospora crassa* has been one of the most important materials for genetic research. During recent years it has been especially valuable in investigations on gene action at the biochemical level. Unfortunately, however, there exists a paucity of cytological knowledge of this ascomycete, which hinders correlations between genetic and cytological data. The most detailed accounts of chromosome morphology and the meiotic division cycle were presented by McCLINTOCK (1945, 1947) and by SINGLETON (1953). These authors, however, observed events occurring in developing asci with no descriptions concerning division phenomena of vegetative nuclei.

Recently TSUDA (1958) and SHATKIN (1959) studied the fine structure of cellular constituents of vegetative hyphae of *N. crassa*, including the nuclei. Their electronmicrographs revealed the nature of the nuclear membrane, but there was no evidence presented for nuclear division. Cinematographic observations of living cells have shown gross cellular behavior but, unfortunately, have not captured the sequences of mitosis because the motion of protoplasm obscured these events (HSU and FUERST 1956; FUERST and HSU 1957).

The first detailed account of the manner of division of somatic nuclei of *N. crassa* was presented by BAKERSPIGEL (1959), who in a series of articles on nuclear division of fungi maintains that "the nuclei do not appear to divide in the manner of classical mitosis. During division individually recognizable chromosomes were not seen to align themselves on a spindle or form a metaphase plate."

The present report describes preliminary observations on nuclear division occurring in the vegetative phase of a normal, wild type strain of *N. crassa.* It also gives an account of the number and morphology of metaphase chromosomes of this species.

MATERIALS AND METHODS

The strain of *Neurospora crassa* employed in this study was Em5256A (EMERSON and CUSHING 1946), a wild type strain which has been carried in our laboratories for a number of years. Crosses involving this strain were used as a standard by McCLINTOCK and SINGLETON in studying the chromosomal sequence occurring in the asci (SINGLETON 1953).

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Mature conidia were harvested from cultures which had been grown on complete agar media in test tubes. A heavy conidial suspension was made in minimal liquid medium (VOGEL 1956). This suspension was filtered through cheese cloth to remove mycelial fragments, and the conidia were allowed to germinate in 50 ml Erlenmeyer flasks in the medium for varying lengths of time at 25°C. Conidia and old hyphae were also fixed directly without germination for comparison. Following the desired period of incubation, the supernatant was decanted after centrifugation, and excess liquid was drained from the tubes. The germinated conidia were then washed thoroughly in distilled water and resuspended in distilled water for at least four hours.

After the water pretreatment the material was recentrifuged and fixed in acetic acid-alcohol (1:3) for 25 minutes. Following fixation the conidia were hydrolyzed in 1 N HCl for eight minutes at 60°C. The cytoplasm of nonhydrolyzed conidia and hyphae stained so densely with orcein that nuclear details could not be critically examined.

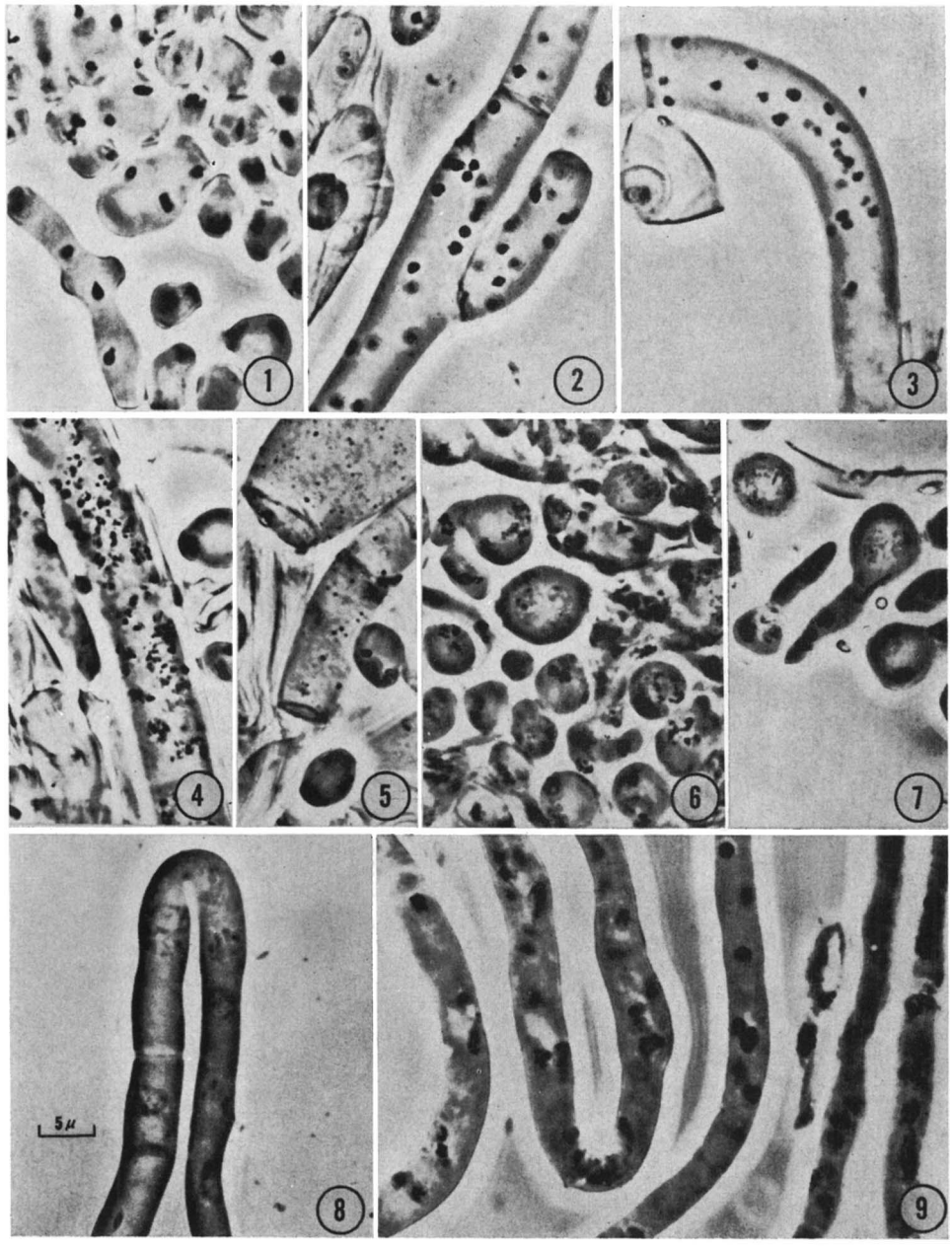
The hydrolyzed material was stained by adding a few drops of stain directly to the material in the centrifuge tubes after HCl was removed. Two percent orcein (Geo. T. Gurr, Ltd., Lot No. 11031) in 50 percent acetic acid was regularly used. However, Feulgen technique was also applied on several occasions for comparison. Squash preparations were made immediately for cytological observations. Storage of such material was performed by adopting the air-dried method suggested by ROTHFELS and SIMINOVITCH (1958) for mammalian cells. These dried preparations could be squashed with fresh aceto-orcein whenever needed.

All observations were made with either a Reichert Zetopan microscope with fixed dark-phase optics or a Reichert Biozet microscope with Variphase optics. For photographic recordings a Zeiss GF-595 microscope was routinely used. This microscope was equipped with Neofluar dark-phase objectives and Leitz Periplan oculars. All photographs were taken with a Leitz Aristophot Model 2 camera and $4'' \times 5''$ Ansco Hypan films. This film is a high speed negative which allows brief exposure time yet preserves good contrast comparable with some films with slower speed, such as Ansco Isopan. All films were developed in Kodak DK-60A developer at 25°C.

OBSERVATIONS

Vegetative nuclei

If mature conidia are harvested and examined directly by the aceto-orcein method just described without having been incubated for a period of time in a suitable growth medium, a variable number of from one to eight nuclei can be seen within each conidium. These dehydrated, "resting" nuclei appear as very small, densely stained bodies approximately 1μ in diameter, which in this extremely condensed state lack all structural details usually visible in interphase nuclei (Figure 1). Nuclei with a similar morphology can be observed in aerial hyphae removed from a mycelial mat which has been allowed to grow for several days in a test tube (Figures 2 and 3). If hyphae are examined from a culture



FIGURES 1–9.—Conidia and mycelia of *Neurospora crassa*, strain Em5256. Fixed with acetic alcohol, hydrolyzed in HCl, aceto-orcein squash. Dark-phase microscopy. Figures 6–9, material treated with distilled water before fixation. For magnification see scale on Figure 8. Figure 1.—Dried conidial spores directly taken from old cultures. Note the compactness of the truly "resting" nuclei, their small size and lack of structural details. Figures 2 and 3.—Resting nuclei in dried mycelia with similar characteristics as those of dried spores shown in Figure 1. Figure 4.—A dried mycelium containing degenerating nuclear material. Figure 5.—A dried mycelium containing disintegrating nuclear material shown as tiny dark spots. Figure 6.—Conidial spores immersed in liquid medium for ten hours. Note various stages of mitotic division. Compare the nuclei in this picture with those in Figures 1–3. Figure 7.—Germinating spores showing hyphal tubes and active nuclei. Figure 8.—A section of a mycelium 24 hours in culture, showing the structure of interphase nuclei. Note the heterochromatic regions in some of the nuclei. Figure 9.—Mycelia 24 hours in culture. Note interphase nuclei and some mitotic figures.

which is several weeks old, evidence of nuclear degeneration and disintegration is noted (Figures 4 and 5). Nuclear remains and fragments are of extremely variable size and shape. Since many of these fragments are within the size limits of chromosomes, it is desirable to avoid examining cultures of this age for evidence of mitosis.

If, however, dehydrated conidial spores are allowed to germinate for several hours in a suitable growth medium, cellular constituents become hydrated and active germination and growth resume. This activity is reflected by a multiplicity of mitotic figures as well as interphase nuclei (Figures 6 and 7). Furthermore, this activity can still be observed after 24 hours of incubation, for there is still an abundance of mitotic figures while interphase nuclei show their characteristic structural details (Figures 8 and 9).

Nuclear division

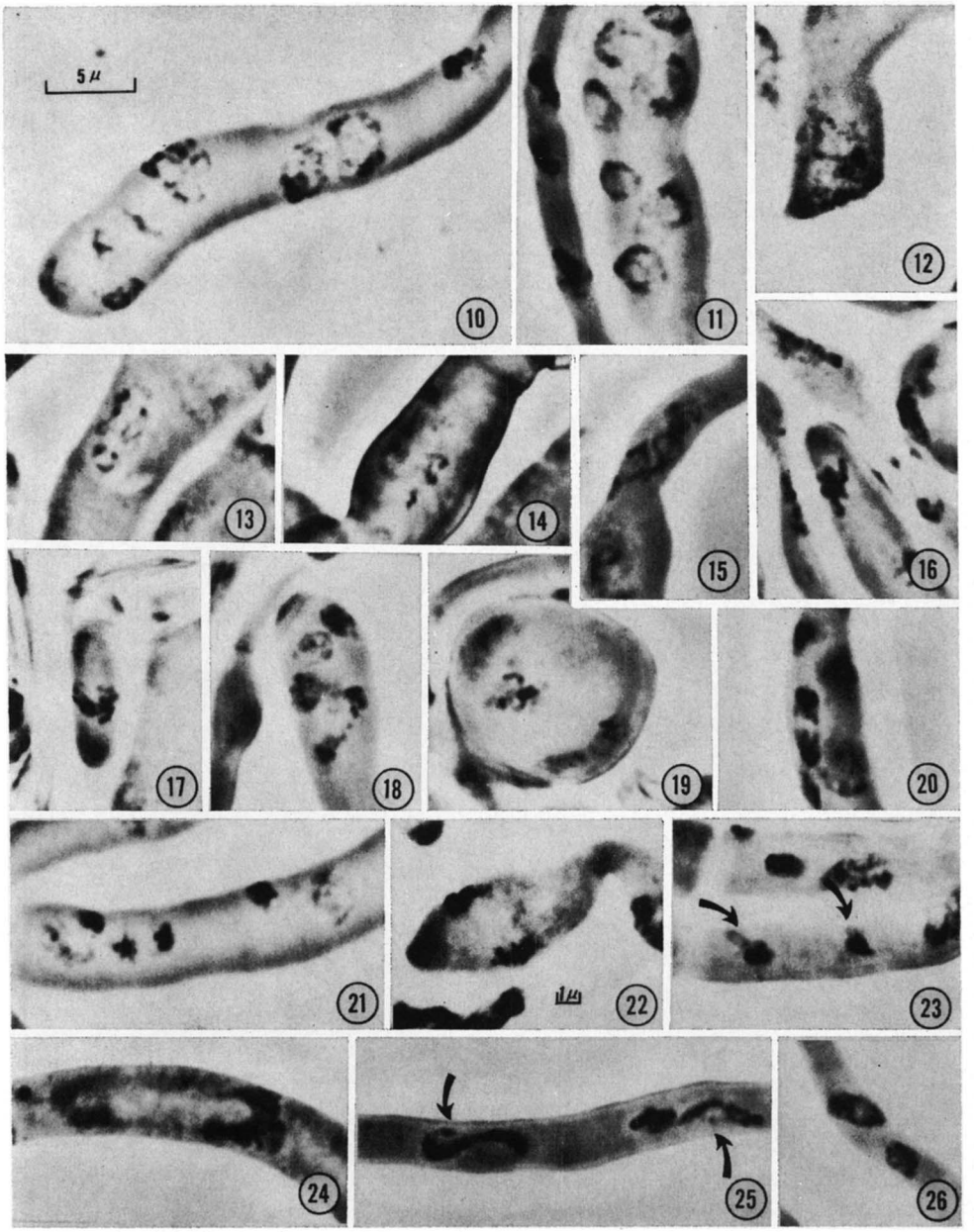
The various stages of nuclear division recorded from these actively growing cultures are represented by Figures 10–26. In Figures 10 and 11 characteristic round or oval interphase nuclei can be seen to vary from 2 to 3μ in diameter. Each nucleus contains a nucleolus and a prominent heterochromatic segment. While there are apparently several small regions of heterochromatin in each nucleus, the major portion is probably contributed by two large heterochromatic segments in chromosome one, the largest chromosome (SINGLETON 1953). These two segments can frequently be observed as a V-shaped figure in interphase nuclei.

Apparently prophase proceeds in the conventional manner (Figures 12–17). The chromosomes in the earliest detectable stages of prophase are more difficult to observe because of their weak stainability (Figure 12). However, while the chromosomes are as yet not highly contracted, the heterochromatin is differentially discernible. As spiralization proceeds during prophase, individual chromosomes can be observed as well as coils within chromosomes (Figures 13 and 14). Later stages of prophase showing increasing contraction of individual chromosomes are represented by Figures 15, 16, and 17.

Chromosomes, when fully contracted, assume configurations similar to regular mitotic metaphase (Figures 18 and 19). When observed from side view, individual chromosomes can be seen to align on a metaphase plate, and from a polar view of this stage morphology of individual chromosomes may be distinguished.

Very early anaphasic figures in which all chromosomes could be counted have not been recognized. However, fairly early anaphase figures have been recorded in which the ends of the longest chromosomes extend from two groups of separating daughter chromosomes (Figure 20). In Figure 21 two anaphases are evident. Observations made on late anaphasic and early telophasic figures suggest the presence of a spindle (Figures 22 and 24).

Formation of nucleoli is readily recognized during telophase, and frequently the nucleolus can be detected at one side of each chromosome group (Figure 23). One aspect of telophase in Neurospora, which seems to differ from similar stages in other organisms, is that during the stage of despiralization of chromosomes



FIGURES 10–26.—Mitotic stages in *Neurospora crassa*, strain Em5256. Treated with distilled water, fixed with acetic alcohol, hydrolyzed in HCl, aceto-orcein squash. Dark-phase microscopy. For magnification for all pictures except Figure 22, see scale on Figure 10. Magnification scale on Figure 22 for this picture only. Figures 10 and 11.—Interphase nuclei in mycelia. Note heterochromatin and nucleoli. Figure 12.—An early prophase nucleus showing a conspicuous heterochromatic segment. Figures 13 and 14.—Prophases. Figure 15.— Late prophase. Figures 16 and 17.—Prometaphases. Figures 18 and 19.—Metaphases. Figure 20.—Early anaphase. Figure 21.—A section of mycelium showing two anaphasic figures. Figure 22.—Late anaphase. Note the image of spindle indicated by the space with low contrast. Figure 23.—Telophase showing the beginning of formation of nucleoli (arrows). Figure 24.—Early telophase. Note the two chromosome groups and the image of spindle indicated by the space with low contrast. Figure 25.—Late telophase, showing the formation of elongated daughter nuclei and oval nucleoli (arrows). Figure 26.—Very late telophase, entering interphase. The nucleolus in each nucleus is now located at the center.

the daughter groups assume a much elongated, instead of the regular roundish, shape. Figure 25 is an excellent example showing the shape of the nuclei, the nucleoli and the chromosomal segments at which the nucleoli attach. The young nucleus seems to contract later and becomes more or less spherical. By this stage the nucleolus is still located acentrically, and the chromatin mass is often displaced to the other side. A pair of daughter nuclei representing very late telophase is shown in Figure 26. The nuclei are entering interphase, and at this stage the prominent nucleoli are centrally located.

Chromosome number and morphology

In the majority of metaphase figures observed, seven distinct chromosomes could be counted (Figures 27–31). However, in approximately one fourth of the figures a tiny eighth chromosome is noted (Figure 32).

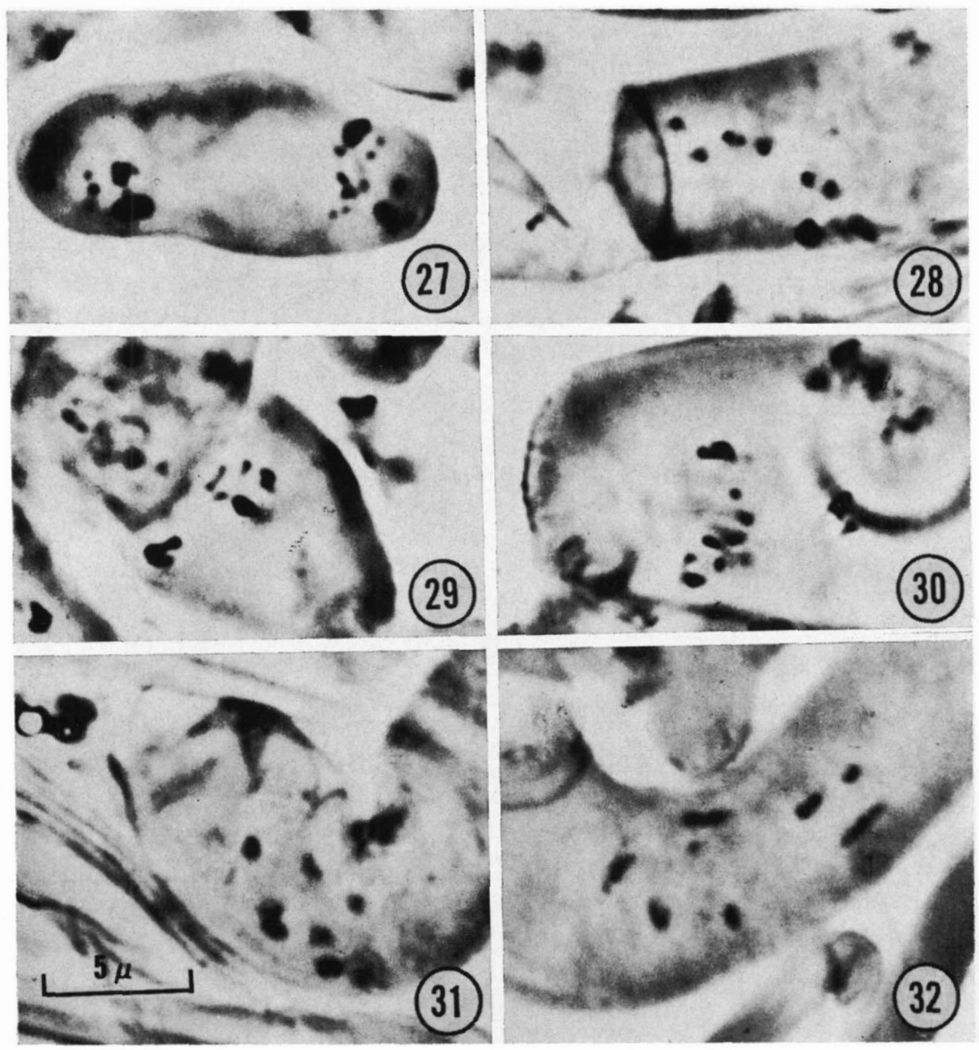
The largest chromosome, chromosome 1, is approximately 1μ in length and is characterized by having a subterminal secondary constriction. Chromosome 2 is slightly shorter in length and possesses a relatively long secondary constriction and a satellite (Figures 27 and 30). The other five major chromosomes vary slightly in length but are all noticeably shorter than chromosomes 1 and 2. These smaller chromosomes are each characterized by a single, more or less median, primary constriction. The smallest, the eighth chromosome, is approximately one tenth the size of the large chromosome 1.

DISCUSSION

The observations reported herein present evidence for division of vegetative nuclei in *N. crassa* which is similar to mitosis of somatic nuclei of other organisms. These observations are not in agreement with BAKERSPIGEL's (1959) report of a seemingly amitotic system in division of somatic nuclei of this fungus. However, it is believed that this difference lies only in interpretation, for the division figures recorded in BAKERSPIGEL's photographs are similar to those presented in this report.

For instance, in the case of interphase nuclei, the "granule" seen by BAKERSPIGEL (Figure 48, 1959) is reported here as a heterochromatic segment. Furthermore, BAKERSPIGEL's Figure 43 appears similar to prophase figures, while his Figure 13 could be interpreted as prometaphase. Similarly, his Figures 12, 14, 52, and 53, which he considers as complexes of chromosomal filaments, are somewhat comparable to metaphase configurations in which chromosomes have not been spread sufficiently widely to show their individual morphology.

In comparing our own preparations made with the Feulgen techniques observed with light microscopy and the aceto-orcein squash viewed with phase microscopy, there was no question that the latter method gave more contrast and revealed more detail. The water pretreatment, the mass culture, and the relatively heavy squashing also contributed to the preliminary success in the study of somatic nuclear division of Neurospora. Nevertheless, critical analysis of the morphology of mitotic chromosomes still awaits further improvement of



FIGURES 27–32.—Chromosomes of *Neurospora crassa*, strain Em5256. Treated with distilled water, fixed with acetic alcohol, hydrolyzed in HCl, aceto-orcein squash. Dark-phase microscopy. For magnification see scale on Figure 31. In Figure 27 a germinating conidial spore is shown with two metaphase figures. The two larger chromosomes on the right side are chromosomes 1 and 2. Chromosome 1 possesses a prominent subterminal constriction, and chromosome 2 has a satellite. Other chromosomes are two-armed. In Figure 30 chromosome 1 is located at the top of the metaphase plate. Note the constriction. Chromosome 2 is at the bottom of the plate. Note the long, secondary constriction which connects the main chromosome body and a satellite. In Figure 32, eight chromosomes can be clearly seen, one being very small.

techniques. Although the spreading of chromosomes is greatly enhanced by the distilled water pretreatment, the cell wall actually limits the dimensional expansion of the protoplasm. Perhaps this difficulty will be resolved by studying chromosomes in protoplasts obtained by the method as outlined by BACHMANN

and BONNER (1959). An attempt is being made to determine whether this could be a possible method for eliminating our present handicaps.

While other fungi have not been observed, it is not unreasonable to suggest that an improved method can be applied to assist in developing the field of cytology of fungi, which for a number of years has had conflicting reports in the literature regarding chromosome numbers and mitosis (MARTENS 1946; CUTTER 1951; OLIVE 1953).

Since it is now feasible to observe chromosome behavior and morphology in vegetative hyphae of Neurospora and to handle relatively large amounts of material for observations, cytological analysis can be made of certain aberrant genetic results obtained by several investigators. MITCHELL, PITTENGER and MITCHELL (1952) and PITTENGER (1954) postulated that the behavior of pseudo-wild types in outcrosses of *Neurospora crassa* was possibly due to an N + 1 chromosomal constitution. More recently, MITCHELL (1958, 1959) has suggested that the aberrant behavior in certain genetic recombinations may be the result of hyperhaploidy with each additional mutant chromosome being a step toward prototrophy. These hypotheses should be testable by cytological observations.

Although the first article on chromosome number of *N. crassa* reported it to vary from six to nine (LINDEGREN and RUMANN 1938), for many years seven has been accepted as the haploid set. This was mainly based upon the observations of MCCLINTOCK (1945) and SINGLETON (1953) on chromosomes in the ascus. Seven is also the number of known linkage groups in this organism (BARRATT, NEWMEYER, PERKINS and GARNJOBST 1954). However, from observations on somatic chromosomes reported here it appears that the certainty of this number is no longer firmly established. While seven also appeared in most of the cells observed in the present study, a very small eighth chromosome has been noted in several metaphase figures (Figure 32).

This eighth chromosome may be a supernumerary that occurs only in a small fraction of the nuclei of the stock employed as in the case described by MCCLINTOCK (1954), or a supernumerary chromosome particular to this strain, or even a regular member of the haploid set of *N. crassa*. With the present technique, it is rather difficult to obtain conclusive evidence because our knowledge about the morphology of the seven larger chromosomes is still far from adequate. Since several larger chromosomes possess secondary constrictions, the smallest chromosome, if it is present regularly, may easily be regarded as a part of one of the larger elements. Another possibility is that the eighth chromosome may be lying above or beneath one of the larger chromosomes so that it is not always visible. It is also possible that the eighth chromosome is largely heterochromatic in nature in addition to its small size (approximately 0.1μ in length). This would probably explain the difficulty in accounting for an eighth linkage group. However, without further improvements in cytological techniques, together with re-examination of pachytene chromosomes in the ascus, the true nature of the eighth chromosome cannot be ascertained.

SUMMARY

1. A method is outlined for the study of mitosis in *Neurospora crassa*, and observations on mitosis in a wild type strain are described.

2. The morphology and number of the metaphase chromosomes are described and discussed. While seven chromosomes are easily distinguished, it is possible that an eighth chromosome also exists.

3. The feasibility of using the method presented herein to study mutant strains of *N. crassa* which show aberrant genetic behavior is discussed.

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