A Study of Mitosis in the Mold Blastocladiella with a Ribonuclease-aceto-orcein Staining Technique

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Introduction

In a recent study of some of the factors controlling the rate of nuclear multiplication and synthesis of deoxyribonucleic acid (DNA) in Blastocladiella emersonii (Turian and Cantino, 1959), we used a formaldehyde-toluidine blue fixation-staining procedure; this facilitated nuclear counts thanks to the intense, violet-blue coloration imparted to the single, large nucleolus or central body of the otherwise pale blue nuclei.

Mitotic figures were easily recognized because of their persistant, elongating nucleoli (also observed in the related mold, Allomyces; Turian 1959), but the intense and uniform basophilic of the whole spindle-shaped body precluded any identification of the chromatin and its disjunction stages at mitosis. Aceto-orcein alone was also useless for this purpose, as already experienced by Sost (1955) with the vegetative nuclei of Allomyces, because of its indiscriminate affinity for both chromatin and nucleolar material.

It was soon realized that proper identification of chromatin—presumably tightly bound around the central body as recently suggested for Allomyces nuclei (Turian 1959)—would first require removal of the highly basophilic constituents (apparently ribonucleic acid (RNA); see results) of the nucleolar material. But instead of employing the acid hydrolytic methods of Feulgen or the HCl-Giemsa previously used for Allomyces (Turian, 1958, 1959) which might have caused artefacts in such delicate, fungal nuclei, we decided to apply a milder hydrolysis with ribonuclease and followed this with an aceto-orcein stain.

Materials and methods

Populations of Blastocladiella emersonii (Cantino and Hyatt 1953) were...
grown for 20–22 hours on plates of peptone-yeast extract-glucose (PYG agar medium of Cantino 1952, now available as Bacto-Cantino PYG agar, Difco Laboratories, Detroit). After flooding the plates with sterile, distilled water, the mature plants discharged their spores quickly; aliquots of the resulting swarmer suspension were added to a shallow layer of fresh, liquid PYG medium on the bottom of small, sterile Petri dishes.

Because of their well-developed and apparently “sticky” rhizoidal system, 3–5 hour germlings adhered strongly to the bottoms of the dishes. Excess nutrient liquid was decanted without dislodging the plants, and replaced by Carnoy’s acetic acid-ethyl alcohol (1:3) fixative. After a 15–20 minute contact with the fixative, the germlings were washed by successive additions of alcohol-water mixtures of gradually decreasing strength. After the final wash with pure water, they were covered with a solution of ribonuclease (2 mg/ml; Sigma Chemical Company.) for 2–3 hours at ca 25°C. Exceptionally good results were obtained when germlings were incubated with the ribonuclease for one hour at 25°C then ca 16 hours at 4°C, followed by a final incubation of one hour at 25°C.

After decanting the enzyme, germlings were gently collected with a rubber scraper in a minimal volume of distilled water and then flooded with an excess of aceto-orcein. After 1 hour, samples of the dense, red germling suspension were transferred to alcohol-defatted slides for microscopic observations. Excess orcein was carefully replaced with dilute acetic acid and this, in turn, with tertiary butyl alcohol before the germlings were mounted in diaphane. Photomicrographs were made with a Bausch and Lomb microscope using a green filter and Adox film.

Results

The method described above permitted the successful resolution of DNA-chromatic material from other basophilic, RNA-containing structures such as the nucleolus. Furthermore, we were able to confirm our notion that the chromatin was tightly appressed along the surface of the nucleolus. Thus, at certain optical levels, our observations of quiescent and “early prophase” nuclei revealed the localization of chromatin in the form of a dense ring (fig. 1 and 7), around the presumably-proteinaceous matrix of the spherical nucleolus (e.g., stage V1; Turian 1959). In most instances, the distinctly-beaded shape of the ring was easily visible.

When, after treatment with ribonuclease, the orcein was replaced with a stain...
having relatively more selective affinities for DNA, such as methyl green (1: 10,000 w/v), the chromatin ring took on a distinct, greenish hue. Similar results were obtained with germlings of *Allomyces* treated in the same fashion (in contrast to the results derived with the Feulgen and HCl-Giemsa techniques; Turian, 1959).

As mitosis proceeds, the chromatin reorganizes in a crescent-shaped form around the still-spherical but enlarged and hypertrophied nucleolus (e.g., stage V₂; Turian, 1959). The crescent shape seems to be due to a unilateral accumulation of chromatic material, as seen in lateral, optical views of the nucleus (fig. 2; 6 and 7r). Thus, a frontal view of the crescent leaves one with the impression that he is looking at an “early anaphase” (fig. 3, 4, and 7r), even though this stage really corresponds to the prophase-metaphase stage of a mitotic cycle. In this crescent state, the chromatin appears to be composed of a reasonably homogeneous matrix containing some denser, chromatic granules (fig. 3). It is important to note, further, that such crescents have also been observed previously among the mitotic nuclei of *Allomyces* stained with the HCl-Giemsa method; their existence has been confirmed in the present study using the ribonuclease technique.

In *Blastocladiella*, as in *Allomyces* (stage V₂; Turian 1959), the elongation of the nucleolus is easily observed with the formaldehyde-toluidine blue method. This elongation appears to represent the fundamental device which provides for the mechanical separation of the two chromatic masses. The
latter appear in "anaphasic" figures (fig. 4 and 5) on the spindle-like, nucleolar remnants. Such "anaphases" are more typical in frontal views (fig. 5, and 7r) than in lateral ones which still reveal a thinning of the middle zone of the original chromatic crescent with some lagging chromatic filaments on the elongated nucleolar body (fig. 5r and 7n). Moreover, in "late anaphases", chromosome-like bodies could sometimes be distinguished among the chromatic masses (fig. 5r).

After thorough extension, the central region of the nucleolus undergoes constriction preceding its quick separation into two, sister nucleoli. This stage of constriction and separation (V4; Turian, 1959) is the most difficult to follow "in vivo"; the process is more easily detected with the formaldehyde-toluidine blue method. As a result of the nucleolar extension, two chromatic masses are pulled far apart such that the picture resembles a telophase (fig. 6) which precedes reorganization into two sister nuclei. At this stage the nuclear membrane, which seems to persist (intranuclear mitosis), apparently is stretched by the two chromatic groups which are moving apart; thus, the central regions of the membrane are brought together in close proximity until, finally, the sister nuclei become separated from one another.

Discussion

Aside from proving excellent visibility of chromatin with a minimum of artifacts, the application of this ribonuclease-aceto-orcein technique to the mitotic nuclei of Blastocladiella has revealed two important facts:

1. The absence of a metaphase plate; the mitotic sequence implies a direct transition from an aberrant type of "prophase-metaphase" (chromatic crescent) to a conspicuous "anaphase-telophase" configuration.
2. The absence of a distinct differentiation into individual chromosomes; the chromatic masses sometimes segment into chromosome-like, denser bodies, but this process does not occur with any real regularity.

The absence of a typical metaphase, along with the absence of individualized chromosomes, is clear indication of an aberrant type of mitosis in the vegetative nuclei of this aquatic Phycomycete, as is also true for its close relative *Allomyces* (Turian 1959). A somewhat similar situation has been described for terrestrial Phycomycetes by Robinow (1957) and Baker-Spiegel (1958). In this type of mitosis, the crescent-like disposition of chromatin at “metaphase” must, in some way, substitute for the absence of a typical metaphase plate. Moreover, it is intimately related to the functioning of the persistent nucleolus which, through its elongation, provides a mechanical device for pulling apart the two, daughter, chromatic masses to opposite poles. Further support for the notion that this peculiar nucleolus takes an active part in vegetative mitosis is derived from its susceptibility to the action of complexing compounds such as boric acid; the latter is capable of interfering with some of its functional components, presumably RNA, with the expected consequence of polyploidization (endo-) as observed in *Allomyces* (Turian 1959).

In contrast with the situation in the reproductive, meiotic nuclei of *Allomyces*, in which Wilson (1952) and Emerson and Wilson (1954) have...
already clearly described and counted individual chromosomes, the mitotic stages of vegetative nuclei in Blastocladiella emersonii as well as in Allomyces, do not reveal clear-cut individual chromosomes. Of course, the possibility must not be overlooked that persistence of the nuclear membrane around chromatic material (intranuclear mitosis) might have prevented normal scattering of the individual chromosomes in these fungi; this too, has also been suggested for other fungi such as yeasts (Ganesan and Swaminathan 1958).

But, even if typical chromosomes really do not differentiate in these vegetative nuclei, we can only assume that some mechanism must still be provided for assuring an equal distribution of genetic material. Endomitotic replication of hereditary material has been suggested by Robinow (1957) for vegetative mitosis in the Mucorales. We have also suggested the possibility of endopolyploidization by uninterrupted mitosis in Allomyces treated with polyploidizing agents (such as boric acid, Turian 1959). Independence of DNA replication at the strand level, on the one hand, from nuclear division in the form of a kind of pseudo-amitosis, on the other, could then be visualized for members of the Blastocladiaceae, as well as other fungi. Recognition of this type of mitosis in the Blastocladiaceae, in particular, permits us to explain, in terms of different degrees of endopolyploidization, the very puzzling, gradual increase in nuclear size which is encountered in the arbuscule mycelium of Allomyces—for example, from its hyphal tips (relatively small nuclei) to its main branches and basal “trunk” (large nuclei). Thus, only small, “normal” nuclei would be enclosed automatically in hyphae at gametogenesis, during which gametangia are differentiated from hyphal tips. Interestingly enough, a rather similar type of primitive mitosis which involves only “anaphase” figures has also been described in liliaceous root cells by Dietrich (1957), who considers them as examples of “pseudo-amitosis” in the sense of Politzer (1934). It may be that this rather direct and unsophisticated type of mitosis, first described for fungi by Leger (1896), is more generally distributed among the vegetative cells of diverse groups of plants than was heretofore suspected.

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### Summary

By combining a ribonuclease digestion of the basophilic constituents in the persistent nucleolus with a subsequent aceto-orcein staining, the mitotic evolution of chromatin in vegetative cells of Blastocladiella was studied.

The following sequence of stages was described: a beaded, chromatin
ring, succeeded by a “prophase-metaphase” chromatic crescent (both around a spherical nucleolus); and then an anaphase-like figure, followed by a “telophase” arrangement (around an elongating and a constricted, spindle-shaped nucleolus, respectively).

Neither metaphase plates nor individually recognizable chromosomes were observed. The suggestion was made that endomitotic reproduction of the hereditary material in this fungus is normally, but not necessarily, followed by pseudo-amitotic, intranuclear division.

References


