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ISOPYCNIC ANALYSIS OF INTACT CELLS - II: CHANGES IN *Aspergillus ornatus* WITH AGE

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SUMMARY: Shake cultures of the Ascomycetes fungus *Aspergillus ornatus* were tested for changes in cellular specific gravity versus aging. The sharp rise in specific gravity in the germinating culture was followed by a slower, but substantial drop as the hyphal cells aged. DNA, RNA, crude protein, and crude lipids were all measured against the pycnotic curve. The decline of the cellular specific gravity is attributed to a substantial rise in lipid content.

Analysis by density gradient centrifugation provides a simple and novel approach for the monitoring of gross chemical changes which can occur as an organism grows and ages. Although there is no consensus among biologists as to the definition of aging, one widely accepted viewpoint was articulated by Strehler (1), and again by Comfort (2). In this view the aging process is defined as the decreasing ability for an ogranism to adapt and to respond to its environment after the onset of sexual maturity. All developmental and functional events preceeding this period are designated as growth, and are of a separate, more positive nature in that they are usually beneficial to the organism, while aging connotes decreased capacilities and deterioration. Ideal situations for the study of aging are often rare, because of the notoriously obscure and complex machinery of a rapidly changing, actively thriving organism; however, some of the aspects of aging in simple organisms have been studied, such as changes in enzymes (3, 4), and changes in biochemical composition with time (5, 6). Density gradient centrifugation avails itself to a more general overview of such biochemical changes, because the technique measures variations in cells, which is actually an attempt to recognize the characteristics of the forest before cataloguing the trees. Thus discovery of any trends in cellular specific gravity can

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then result in attempts to correlate what is occuring at the macroscopic level with that at the subcellular level by chemical tests which can qualify the relative amounts of the major cell constituents.

Whereas in our first paper (7) we reported on the ramifications of specific gravity changes in a unicellular prokaryote, we here wish to examine a relatively simple multicellular eukaryote. Observations on the homogeneity of individual cultures, and the role changes in certain cellular components may play in our hypotheses on the progressive deterioration of the organism.

MATERIALS AND METHODS

Aspergillus ornatus cultures were maintained on Schwemmin's complex medium agar slants kept in the dark at 4C (8) until use in liquid medium. Flasks of liquid medium set at pH 4.8 were inoculated with small pieces of mycelia and placed on a reciprocal shaker at facilitate thorough mixing, aeration, and growth of hyphae into spherical globules.

Isopycnography was done on small (approx. 30 mg) portions of mycelia that were excised from each globule. This was added to 5 ml distilled water in a microblender for 10 seconds for purposes of disaggregation. Density gradients of NaBr in water were prepared according to the method reported in our first paper (7). Each gradient ranged from 1.2 to 1.5 gm/cc. Two milliliters of the mycelial suspension were layered atop each gradient, which were then centrifuged at 10,000 x G for 10 minutes. Specific gravities of the resultant bands were determined refractometrically.

Biochemical tests for dry weight, DNA, RNA, protein, medium glucose and pH were periodically made. Mycelia were removed from each flask, dried at 55C for 48 hours and weighed. DNA and RNA determinations were made by pooling the dried mycelia from replicates and using the diphenylamine and orcinol techniques (9). The Folin phenol reagent method (10) for protein quantification was used, and the glucose concentration in the medium was followed by the 3,5-dinitrosalicylate method (11). Lipid quantification of both whole cells and the cell walls was done according to the methods of Hunter and Rose (12).

Tests for intraglobular homogeneity required pycnographic analysis of small portions of mycelia taken from 4, 8, and 12 day cultures and from both inside and outside regions of the globules.

RESULTS AND CONCLUSIONS

During the 12 day course of observations, the pH changed from 4.8 to 4.5, and the glucose went from 40 to 32 gm/liter. These data indicate adequate buffering and carbon source in the medium. Likewise very little variation was seen in specific gravity between samples taken from various re-

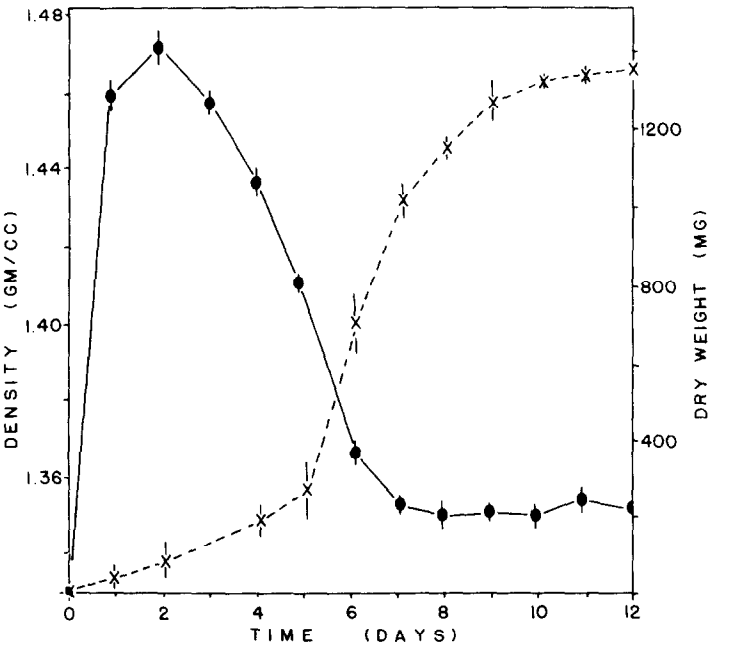

Figure 1. The total dry weight (-x-) and density (-•-) of Aspergillus ornatus vs. age of mycelium. Each of the dry weight points represents the mean of three liquid-grown samples, with the vertical lines representing the standard deviation among the samples. Each point on the density curve is the average value for six runs for each day. The standard deviation for each value on the density curve is represented by a vertical line.

gions of any given globule (±0.04 gm/cc). This implies that there is free flow of nutrients throughout the globule or that there is active transport of relevant components between the member cells, which are linked by perforated septa (3).

As seen in Figure 1, there is a distinct drop in density of the liquid-grown cultures beginning at day 4, with a culminating plateau starting at day 7. This leveling-off effect is seen in the dry weight, DNA, RNA and protein curves as well, suggesting that the organism has reached the stationary phase. If one were to look at mycelia grown on Petri plates, it would be noted that this period is indeed after the phase of sexual maturity (see 3), and can thus be considered as the onset of aging in this organism. Sexual maturity is not visibly evident in liquid-grown cultures because sporulation of the fungus requires an air/surface interface not allowed in a sub-

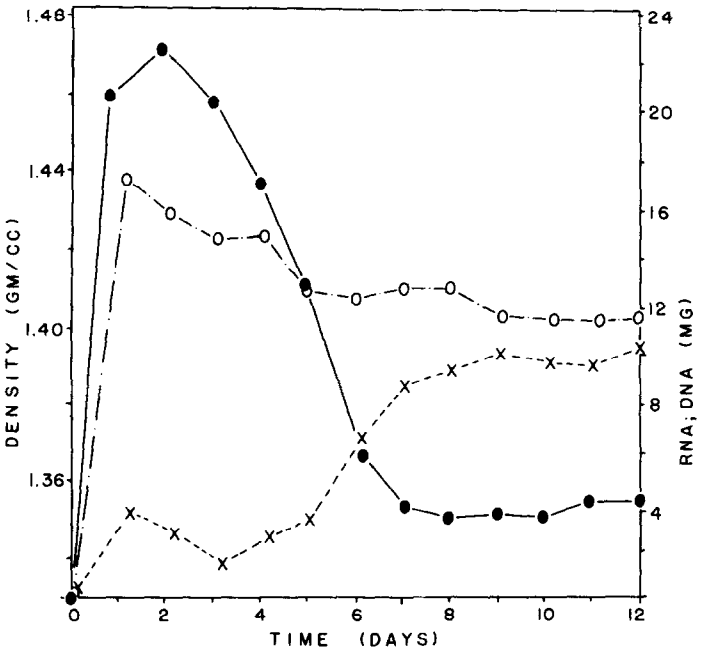

Figure 2. The density curve (-●-) from Aspergillus ornatus cultures is superimposed upon the curves for measuring relative amounts of nucleic acid compounds. The plateau seen after seven days in the density curve is also evident in the DNA (-x-) and RNA plots (-o-). Each of the DNA and RNA points measures the total extracts from three pooled samples for each day.

merged culture; however, the time period for this phase has been described in other studies. While a decrease and leveling off of the heavier elements of the cell is evident, the lipid extracts reveal that there is a marked increase in lipids with age. This escalation of a more buoyant cellular component supports the findings of the specific gravity curve. It was additionally seen that approximately 65% of the lipids attributed to the cells were found in the walls in both the 4 and 12 day cultures.

DISCUSSION

It is due primarily to increasing concentration of lipid in aging cells of Aspergillus ornatus that the cells lose specific gravity, whereas we have already seen in the first report of this series (7) that such changes in the single celled prokaryote E. coli are likely due to variation in RNA content. There have been many other reports of changing compositions within organisms

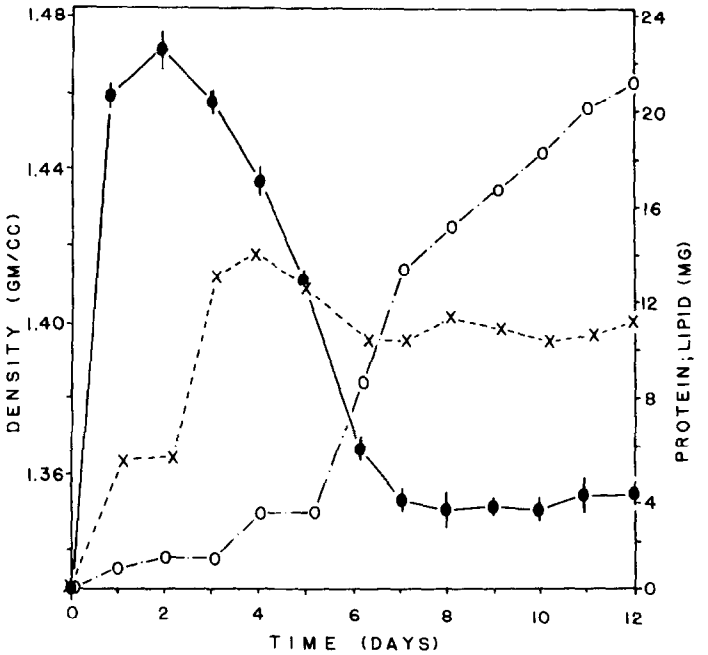

Figure 3. The density curve (-•-) from Aspergillus ornatus cultures is superimposed upon the curves which measure the relative amounts of protein (-x-) and lipid extracts (-o-) in the cells. The plateau which exists after seven days, considered to be the onset of the stationary phase in the organism, is evident in the protein curve but not in the lipid curve. The decrease and subsequent plateaus of the heavier nucleic acid and protein elements, with the concomitant increase of the lighter lipid components may be considered supporting evidence for the hypothesis of the increasing lightness of Aspergillus ornatus cells with age.

with age (13, 14). Many suggest that it is this alteration of constituents which can cause the decreased selectivity of the organism. We have also seen that the increased amount of lipid accumulates in the hyphal walls as the cells age. These sites of accumulation could suggest several possibilities. One is than an increased number of lipids in the wall could lead to a decreased integrity of wall function, where fatty acid chain accumulation could block cell openings or alter conformations of receptors and other proteins in the cell wall appreciably. The other possibility is that the real damage is incurred as a result of the accumulation of lipids in the cytoplasm. It is well-known that fungal cells often accumulate vacuoles with age (15), and that the membranes of these vacuoles are often composed

of lipids to some degree. This increase in vacuolization could be preparing the cell for some aspect of aging or autolysis (16). However, it is also possible that the effects of lipid accumulation can be felt in both the cytoplasm and the hyphal wall, and that many factors beyond these simple hypotheses contribute to the organism's ultimate deterioration. Although there can be no clear-cut determinations of what actually causes aging by this study, density gradient centrifugation analysis does provide a general statement against which other fractional analyses may be compared.

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