# SYNCHRONOUS MITOCHONDRIAL DIVISION IN *NEUROSPORA CRASSA*

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## ABSTRACT

Samples of mycelium of *Neurospora crassa* of known age were harvested from agar plates and examined with the electron microscope. The relative volume of the mitochondria was determined for mycelium of different ages. The volume measurements indicated that the mitochondria were dividing synchronously in fronts 6, 13, and 22½ hr behind the growing hyphal tips. The sequence of mitochondrial division is hypothesized to include mitochondrial cupping followed by division which results in closely associated daughter mitochondria. On the basis of percentages of mitochondrial cupping and association, mitochondrial division was postulated to be occurring at 6, 14, and 26 hr. Close agreement between the mycelial mass doubling time and the calculated mitochondrial mass doubling time indicates that synchronous mitochondrial division is sufficient to maintain growth. The possibility that mitochondrial division is due to intercellular regulation of a mitochondrial genetic system is advanced.

## INTRODUCTION

Luck (9–11) has shown that the increase in the size of the mitochondrial population during growth in *Neurospora crassa* occurs by division of preexisting mitochondria, a finding supported by similar investigations of the common meadow mushroom *Agaricus campestris* (Vogel and Kemper, reference 22) and *Sphaerocarpus donelli*, a liverwort (Diers, reference 6).

Although mitochondria undoubtedly replicate, at least in part, by fission, the actual process is not understood. Bahr and Zeitler (2) interpreted the dumbbell mitochondria they found in rat liver to be division figures and suggested that asymmetric dumbbell ("compound") mitochondria divided into unequal daughter mitochondria. Lehninger (8) suggested that mitochondria seen in an extended dumbbell configuration might be beginning fission. Claude (5) and Diers (6) have come to the same conclusion after studies with rat liver and *Sphaerocarpus* cells, respectively. Stempak (20) observed, in livers from neonatal rats, mitochondria with constrictions, but found upon serial sectioning that these dumbbell configurations were really central sections through a disc shaped like a red blood cell. Weiss (25) and Bell and Mühlethaler (3) found similar mitochondria in rapidly growing cells of *Neurospora crassa* and developing fern egg cells, respectively.

Investigators have variously called these mitochondria dumbbell, "umbo-like", cup-shaped, or ring-shaped. Although among different organisms the dumbbell mitochondria are heterogeneous in appearance, they have an over-all resemblance. In addition, many of the tissues in which dumbbell mitochondria are found are in a state of rapid growth. Since mitochondrial division must keep

pace with cell growth, growing cells might be expected to contain mitochondrial division figures. Dumbbell and cup-shaped mitochondria may therefore represent beginning stages of division. Furthermore, terminal stages of division, before complete separation of the daughter mitochondria occurs, should be expected to include mitochondria in intimate association with each other. Other investigators have indeed seen such mitochondria in association and have interpreted them as being in division. Bahr and Zeitler (2) published photographs of rat liver mitochondria joined by narrow bridges. Those authors interpreted these figures as terminal stages in the mitochondrial division process. Lafontaine and Allard (7) have presented electron micrographs which show mitochondria developing transverse partitions appearing to be double membranes in nature which are fused with the inner mitochondrial envelope. Other micrographs, interpreted as having been taken later in the division process, show slight pinching of the mitochondria at the level of the partition and separation of the transverse partition into two distinct leaflets. These phenomena have been observed in rat cells in which a rapid increase in the number of mitochondria is taking place. Vogel and Kemper (22) have observed such configurations of mitochondrial association in electron micrographs of mitochondrial cultures from *Agaricus campestris* in which proliferation of the mitochondria was apparently occurring. They posited that aggregation seemed an unlikely cause of these cytological figures.

A cytological study was undertaken in this laboratory to explore mitochondrial division in growing *Neurospora crassa* cultures by studying thin sections of cells of known age with the electron microscope. It has been possible to show that the mitochondria in *Neurospora crassa* apparently divide synchronously in specific regions of the growing mycelial mat.

## MATERIALS AND METHODS

### *Neurospora Crassa Strains*

KJT1960a (Wagner and Bergquist, reference 24), a segregant from a cross between the Emerson wild type strains Em5256A and Em5297a, was employed throughout the following experiments.

### *Growth Conditions*

Mycelium was grown on Petri dishes in such a way that the age of the mycelium at the time of harvesting was known. Plates of minimal agar (Vogel, reference 23) were poured and allowed to cool. Wet, sterile circles of dialyzing membrane (Auto Analyzer, Type C, Dialyzing Membrane, Technicon Instrument Corp., Ardsley, N. Y.) were overlaid on the agar. The plates were inoculated by placing a mycelial fragment on a marked spot at their centers and were incubated at 25°C. As growth proceeded, the membrane kept the mycelial pad free of the agar. The edge of the growing colony was outlined on the bottom of the Petri dish every few hours, so that the age of the mycelium was known in the different regions of the pad. Pieces of mycelium of known age were cut out with a razor blade at the same time and fixed for electron microscopy. The age spread of cells within each specimen taken was about 2 hr.

### *Electron Microscopy*

All mycelial samples were fixed, after water rinsing, with unbuffered 2% aqueous $KMnO_4$ for 3–4 hr at room temp. The samples were then stained with 0.5% aqueous uranyl acetate for 16–20 hr at room temp. The samples were dehydrated through an ethanol series to 100% ethanol. The ethanol was then replaced with acetone. The samples were then carried through four plastic resin–acetone mixtures of increasing resin concentrations of 25, 50, 75, and 100%. Samples remained in each resin–acetone change for about 2 days. The plastic resin was a mixture consisting of 10% Epon 812 (Shell Chemical Co., Division of Shell Oil Co., N. Y.), 20% Araldite 6005 (R. P. Cargille Laboratories, Inc., Cedar Grove, N. J.), and 70% dodecenyl succinic anhydride (DDSA) (Cargille Laboratories). Each plastic mixture contained the catalyst DMP-30 (Rohm and Haas, Philadelphia, Pa.). After evacuation and infiltration in 100% plastic, polymerization was brought about by heating for 24 hr at 80°C.

Silver sections (approximately 600 A) were cut on a Servall Porter-Blum MT-1 ultramicrotome with glass knives. All sections were poststained, at this point, with Reynolds' (18) lead citrate for 5 min. Sections were observed and photographed in a Siemens Elmiskop I operated at an accelerating voltage of 60 kv.

### *Analysis of Micrographs*

Organelle cell volume percentages were obtained by a method which is based on the fact that the percentage area occupied by any cellular inclusion in a random section of a cell approximates the percentage volume within the whole cell. The procedure was to trace the mitochondrial, nuclear, and cellular outlines on blank paper, to cut out and separate the tracings of the organelles, and to weigh these pieces of paper for each magnification. Total cell area was determined with a set of standard

ellipses or by graphically subdividing the cells into regular figures, the areas of which could be calculated. The areas were corrected for magnification, were summed for each specimen, and were finally expressed in square microns.

The cytoplasmic volume was obtained by subtracting the nuclear volume from the total cell volume. The percentage of the cytoplasmic volume that was occupied by mitochondria (mitochondrial cytoplasmic volume percentage, MCVP) was obtained by dividing the mitochondrial volume by the cytoplasmic volume and multiplying by 100.

The average cross-sectional area of each mitochondrion was easily obtained by dividing the total mitochondrial area by the number of mitochondria examined within each specimen.

A relationship between mitochondrial cross-sectional area and mitochondrial volume was established. The data as first obtained gave the area of the average random section of a mitochondrion. In order to obtain a regular relationship, it was necessary to assume constancy of shape. The relationship for the simplest case, the sphere, was established to be $V = 1.382\sqrt{\bar{A}^3}$ (equation 1), where $V$ is the volume of the whole sphere and $\bar{A}$ is the area of a random section. Although this equation would not be valid if the shape

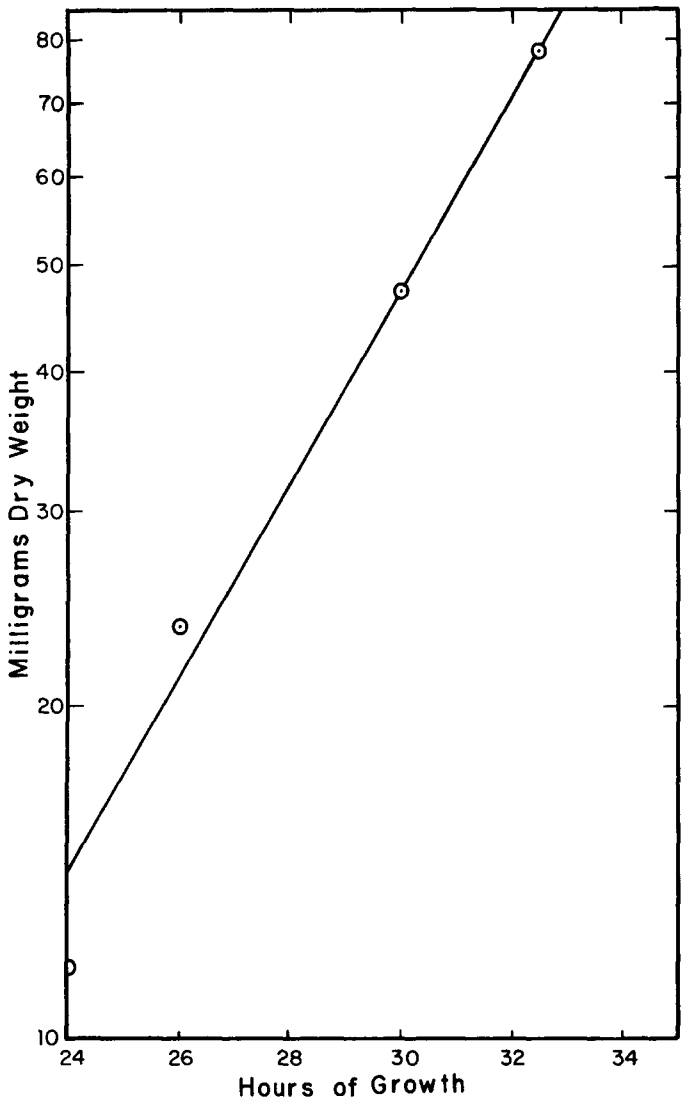


FIGURE 1 Growth rate of *Neurospora crassa*, wild type strain KJT1960a, on dialysis membrane on minimal agar. See Materials and Methods.

of the mitochondria were anything but spherical, the only change would be in the constant. The volume would then remain proportional to the square root of the cube of the average area of the mitochondrial sections, regardless of the shape of the mitochondrion, as long as the shape was constant. Not only would this relationship remain true as long as all mitochondrial shapes were the same, but it would still be valid if there were different shapes in the samples, as long as the proportions of the shapes remained the same.

## RESULTS

Wild type mycelium of known age was harvested after 35 hr of growth from a minimal agar plate overlaid with dialysis membrane and was then fixed for electron microscopy. Seven samples were harvested at the same time from regions of a single mycelial pad known to be 4, 8, 12, 16, 20, 24, and 30 hr old.

A growth study was also performed. Plates were grown as for electron microscopy, and the whole

TABLE I

*Changes in Mitochondrial Area and Configuration as a Function of Mycelial Age*

| Age | MCVP* | Mitochondrial area | Mitochondrial‡ vol. | Relative vol. | Cumulative rel. vol. | Associated mitochondria | Dumbbell mitochondria |
|---|---|---|---|---|---|---|---|
| *hr* | | $\mu^2$ | $\mu^3$ | | | % | % |
| 4 | 10.6 | 0.231 | 0.153 | 2.71 | 2.71 | 4.0 | 7.5 |
| 8 | 13.4 | 0.182 | 0.107 | 1.90 | 4.54 | 10.5 | 1.0 |
| 12 | 11.3 | 0.260 | 0.183 | 3.24 | 5.88 | 0.0 | 19.0 |
| 16 | 11.8 | 0.180 | 0.106 | 1.87 | 7.15 | 20.4 | 0.0 |
| 20 | 11.1 | 0.264 | 0.187 | 3.32 | 8.60 | 0.0 | 2.6 |
| 24 | 11.8 | 0.136 | 0.069 | 1.22 | 9.14 | 0.8 | 0.8 |
| 30 | 13.3 | 0.187 | 0.112 | 1.98 | 9.90 | 18.5 | 1.5 |
| Avg | 11.9 | | 0.131 | 2.30 | | 7.7 | 4.6 |

* Percentage of cytoplasmic volume occupied by mitochondria
‡ Assuming sphericity

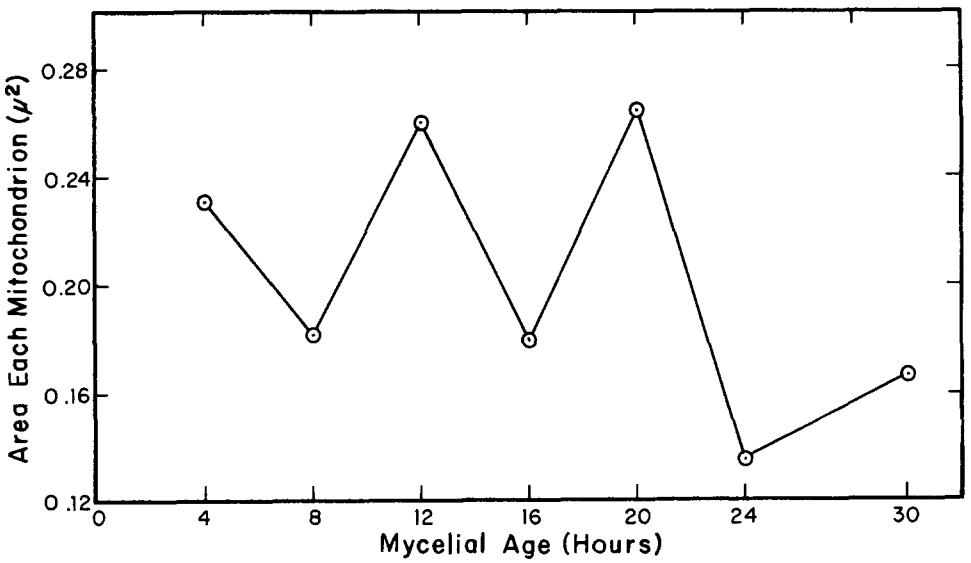


FIGURE 2 Area in square microns of mitochondria in *Neurospora crassa* mycelial fragments of different ages.

mycelial pad was harvested from different plates after 24, 26, 30, and 32½ hr of growth. The mycelium was dried and weighed. The growth study results are plotted in Fig. 1. Mass doubling time under these conditions was about 3.4 hr.

Quantitative data were obtained from electron micrographs for the percentage of the cytoplasmic volume occupied by the mitochondria (MCVP) and the average mitochondrial area. These data are shown in Table I. The average mitochondrial area for each sample was determined from about 240 mitochondrial cross-sections. The MCVP remained relatively constant at about 12%. The mitochondrial area, however, varied considerably. Fig. 2 shows a plot of mitochondrial area versus mycelial age.

When a least squares straight line designed to show a trend in mitochondrial area was calculated for Fig. 2, it was found to be insignificant; therefore, the fluctuations in mitochondrial area were assumed to be real. Since the mycelium was growing, since the MCVP remained constant, and since the mitochondrial size remained within defined limits, the mitochondria were undoubtedly dividing. The mitochondrial area data indicate that this division occurred in a nonrandom manner.

It was hypothesized that division occurred in regular cycles. The sequence of the cycle was assumed to include enlargement of the mitochondria, throughout the course of the cycle, which was followed by mitochondrial division and, as a consequence, an abrupt drop in the volume of each mitochondrion. The cycle would then begin again.

The range of a division cycle was calculated as follows from the mitochondrial area values. The average volume of the mitochondria from all the samples was determined to be 0.131 $\mu^3$, by using equation 1 and assuming sphericity. The absolute deviation of each sample from this value was calculated; the average deviation was 0.0373 $\mu^3$. Doubling this figure gave the maximum absolute deviation, or 0.0746 $\mu^3$. In the real cycle, this amount of deviation would exist on either side of the mean; the total range of mitochondrial volume in the division cycle would then be 0.149 $\mu^3$. If a division cycle were observed in electron micrographs of sequential specimens, the average mitochondrial volume would increase over a range of 0.149 $\mu^3$ from the first to the last sample studied within one cycle. The lowest mitochondrial volume observed would be 0.0746 $\mu^3$ less than the average, or 0.057 $\mu^3$; the highest value obtained would be 0.0746 $\mu^3$ greater than the average, or 0.206 $\mu^3$.

Since mitochondria are not spherical, the volume values were converted to relative volume. The minimum mitochondrial volume calculated above, 0.057 $\mu^3$, was made a standard unit volume (given an arbitrary value of 1.0). All the other values were expressed as fractions of the minimum. A mitochondrion of maximum size would be 3.64 times the minimum volume, and the range of a division cycle would then be 2.64 unit volumes.

A division cycle was fitted to the mitochondrial relative volume data as follows. When a value for the mitochondrial volume was lower than the one for the next youngest specimen, it was postulated that a mitochondrial division had occurred in the interim growth period. In order that the total increase in mitochondrial volume with age could be plotted, the range in relative volume of a single division cycle (2.64 unit volumes) was added to all of the volume values for specimens of greater age than the age at which division occurred. The volume values for samples taken after the first division had occurred were plotted as being 2.64 unit volumes larger than actually measured. In like manner, other values were also plotted. In Fig. 3, the cumulative increase in the relative mitochondrial volume is plotted against age as explained above. The horizontal lines represent the mitochondrial volume at each division. From this plot we can obtain graphically the age of the mycelium when the mitochondria divide.

The cycle represented in Fig. 3 was superimposed upon the values already shown in Fig. 2, but corrected to relative volume. Fig. 4 is the cycle obtained from the plot in Fig. 3. An average mitochondrial division time of 8.3 hr was obtained for the first two complete cycles of division.

A cytological confirmation of the indicated division cycle was undertaken. In many of the cells studied, cup-shaped mitochondria (Fig. 5) and mitochondria in association (Fig. 6) were observed. Fig. 5 shows the correspondence between a cup-shaped mitochondrion sectioned vertically and a cross-section of the mitochondrial cup. An extended dumbbell configuration is also visible in Fig. 5. The associated mitochondria were delineated one from the other by only a darkly staining line which had no apparent ultrastructure. The line did not show the characteristics of other mitochondrial membranes. These interface lines can be seen in Fig. 6. The percentage of mitochondria in dumbbell configuration and in intimate association was determined for each age

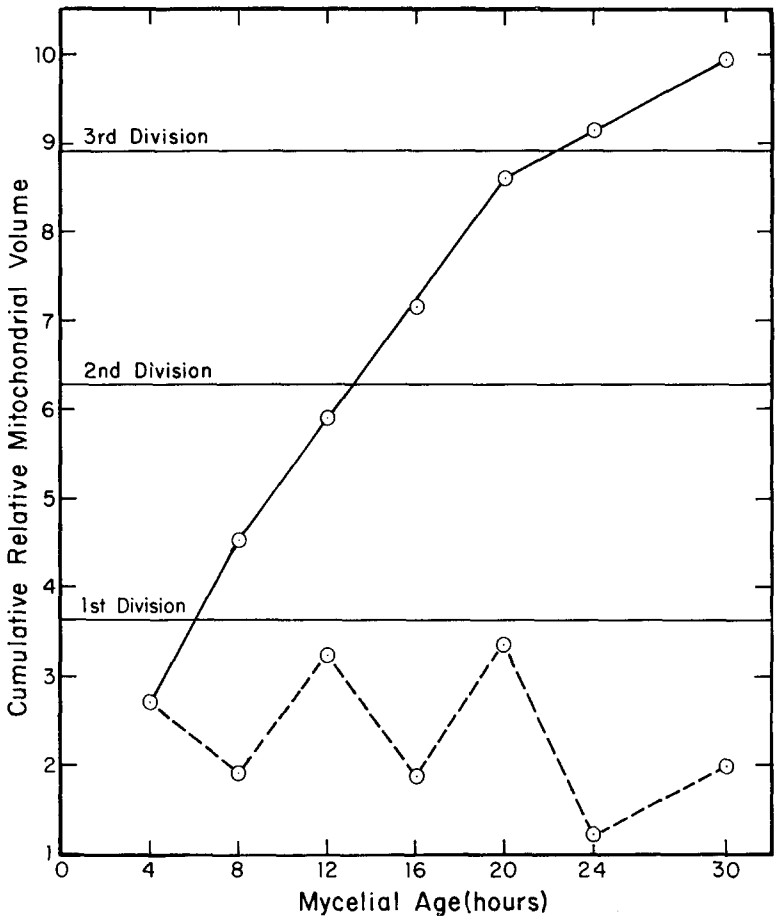


FIGURE 3 Cumulative increase in the relative mitochondrial volume in *Neurospora crassa* mycelial fragments of different ages. The horizontal lines represent the mitochondrial volume at division. The actual relative values of mitochondrial volume (dashed line) are all plotted with a base line higher each cycle by the range of one hypothetical cycle of division (solid line). The change in relative volume of a mitochondrion during one cycle, 2.64, is the difference in volume between the smallest and largest average mitochondrion found during a single division cycle.

specimen (Table I). These values were plotted (Fig. 7).

A division cycle was hypothesized. It was assumed that at the initiation of the cycle small-sized mitochondria would begin to enlarge and take on dumbbell and cup-shaped configurations. As division occurred, the mitochondrial association figures would appear and increase in number as the number of cup-shaped mitochondria decreased. By the time the cycle was at the beginning point again, the bonds of association would be broken.

A regular cycle was fitted to the association and cupping plot in Fig. 7 by using the rationale outlined above. A division was assumed to have occurred in the age interval between any two samples in which the younger sample possessed predominantly cup-shaped mitochondria and the older possessed predominantly associated mitochondria. The length of the first cycle of division obtained in this manner was 8 hr and the length of the second cycle was 12 hr. These values correlate well with cycles of division of 7.0 and 9.3 hr suggested by the volume data, thereby confirming the possible synchrony of mitochondrial division.

## DISCUSSION

Although no detailed study of the cytology of mitochondrial division was undertaken, a few

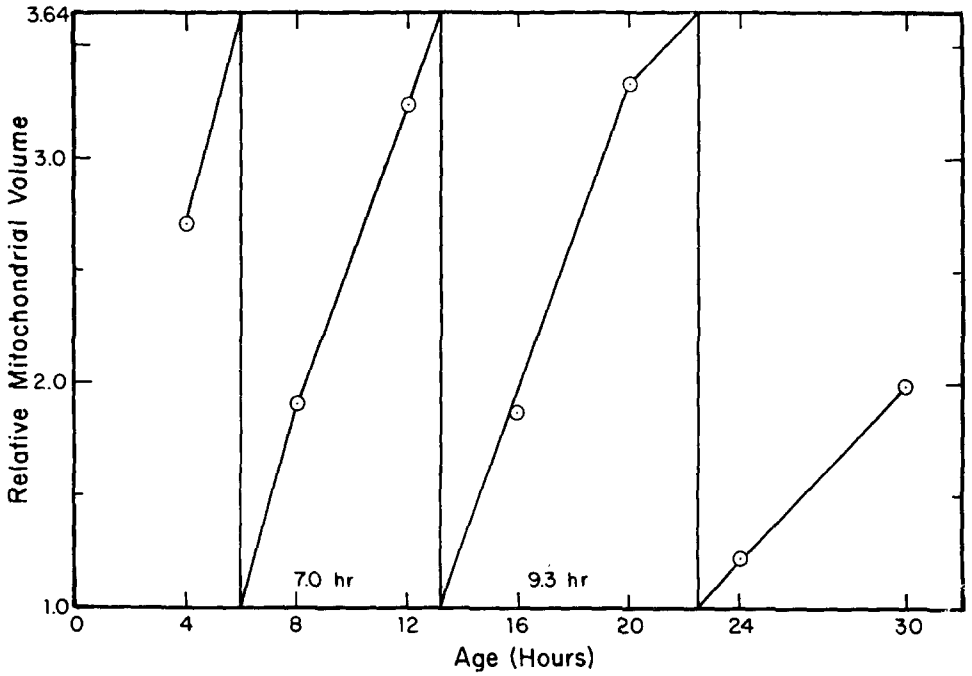


FIGURE 4 Relative volume of each mitochondrion in *Neurospora crassa* mycelial fragments of different ages. A hypothetical division cycle was fitted to the mitochondrial volume data. This plot is the cycle that would be obtained from the curve in Fig. 3.

comments on this process can still be made. A very high degree of reciprocality between the numbers of cup-shaped and associated mitochondria was observed in most samples studied (see Fig. 7). Furthermore, the predominance of one mitochondrial form alternated with the other in time. Moreover, the average volume of a mitochondrion was fluctuating in a manner suggestive of division at the same time as, and correlated in time with, these cytological changes. This suggests that these two mitochondrial forms are mutually exclusive stages of a continuing process. The samples were taken from rapidly growing cultures in which the percentage of the cytoplasmic volume occupied by mitochondria (11.9%) remained constant (Table I). Since division has been shown to be the method of increase in the number of mitochondria (Luck, references 9, 10), it is likely that these two mitochondrial forms represent different stages in the mitochondrial division cycle.

The actual cytological events that occur in the transition from cup-shaped to associated mitochondria are unknown. Since no figures other than rounded, ellipsoid, cup-shaped, or associated mitochondria have been observed in any electron micrographs, either there are no other forms to be observed or else the actual process of division is simply so quick that not enough mitochondria have yet been observed to see the transition state.

The ages at which mitochondrial division occurred, as shown by both the volume and cytological methods of determination, correlate well for the first two divisions. The first division occurs at 6 hr of age as determined by both methods and at 13.2 and 14 hr for the second division as determined by the volume and cytological methods respectively. However, the mycelial age at which the mitochondria divide the third time is left in doubt by a comparison of the two sets of data. The volume plot suggests mitochondrial division at 22.5 hr of age, whereas the association versus cupping plot suggests division at about 26 hr. This latter evidence is not clear-cut, however, because of the ambiguity of the cytological data from 24-hr-old mycelium. The time of persistence of the cytological forms is not known, and it is possible that division figures were missed in the 6-hr sampling pause from 24 to 30 hr. Also, as the rate of mitochondrial division decreases in the older mycelium (see Fig. 3), the degree of synchrony

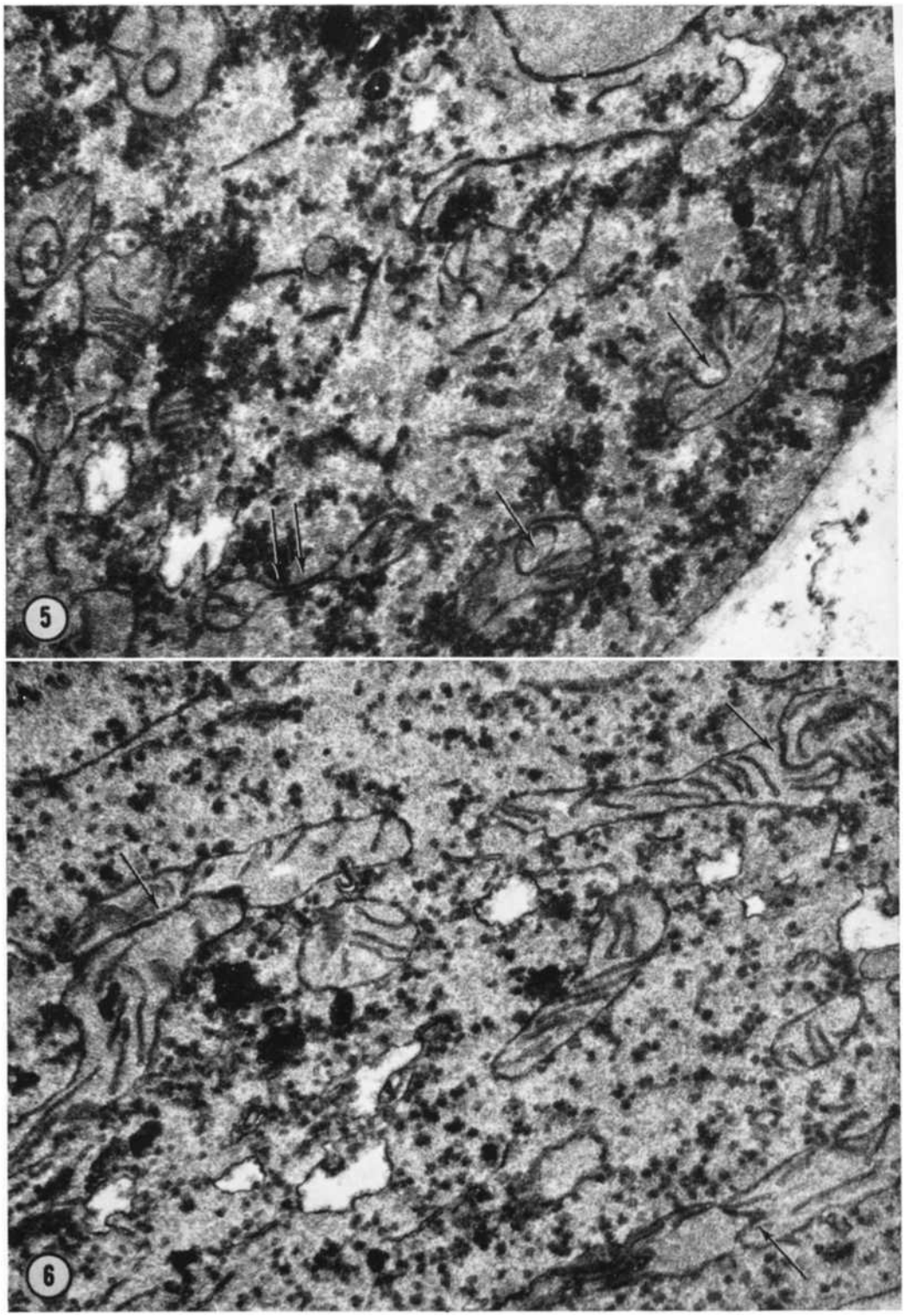


FIGURE 5 *Neurospora crassa* cell, wild type strain KJT1960a, 12 hr old, grown on a minimal agar plate overlaid with dialysis membrane. This cell shows cupped and dumbbell mitochondria. A mitochondrion in a typical extended dumbbell configuration is shown by the double arrow. The equivalence of mitochondrial invaginations in cross- and lateral sections is indicated by the single arrows. × 30,000.

FIGURE 6 *Neurospora crassa* cell, wild type strain KJT1960a, 16 hr old, grown on a minimal agar plate overlaid with dialysis membrane. This cell shows many associated mitochondria. The arrows point to the heavily staining lines which separate the associated mitochondria. × 33,000.

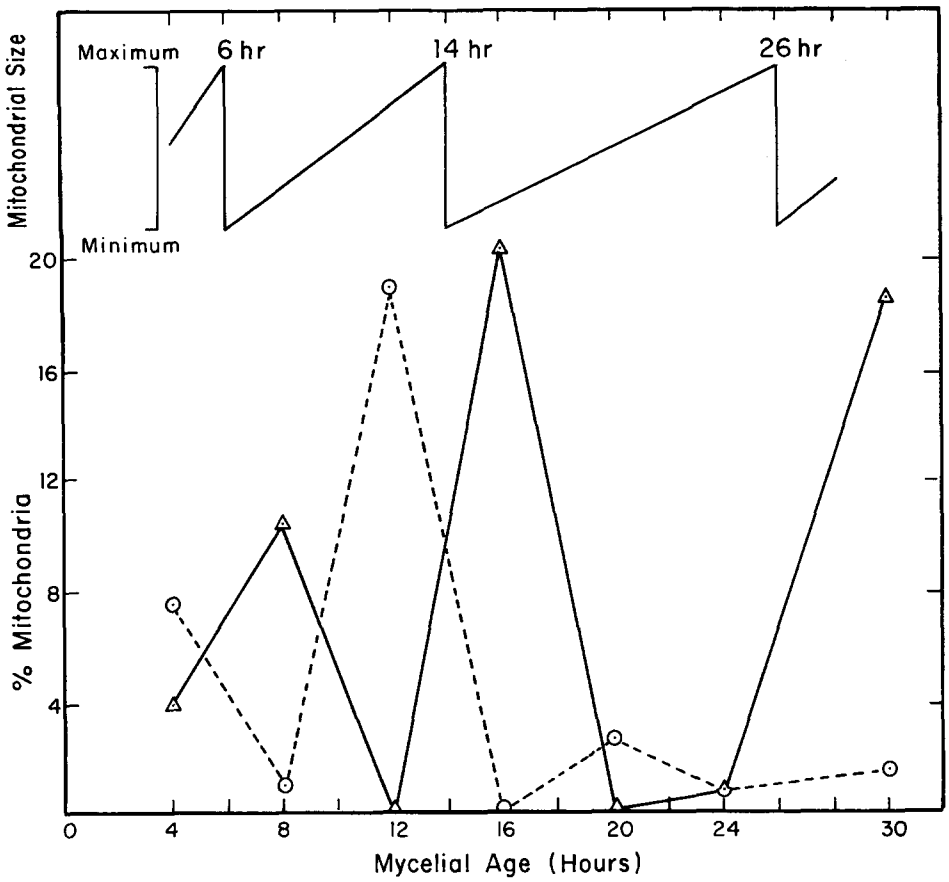


FIGURE 7 Mitochondrial cupping, association, and a derived division cycle in *Neurospora crassa*. The graph at the bottom compares the per cent of mitochondria in association (solid line) with the per cent of mitochondria that are cup- or dumbbell-shaped (dashed line) at different ages The graph at the top is a representation of the division cycle derived from the data shown in the bottom graph.

might drop, and thereby lead to ambiguity in the cytological data.

The division cycles obtained from the two types of evidence presented have been drawn to represent a saltatory division process. However, the sample size was such that a mycelial age spread of approximately 2 hr was represented in each specimen fixed. A cycle can be drawn by integrating the values of a sawtooth cycle over 2-hr periods. Such a cycle shows increasing and decreasing slopes. Furthermore, the total range of the integrated cycle (the one that would be observed) is 0.72 times the range of the sawtooth one it represents.

A question logically raised by the theory that this is a mitochondrial division cycle is whether the cycle can account for the necessary mitochondrial replication needed for cell growth. In the course of one of the proposed mitochondrial division cycles the average mitochondrial volume would increase 3.64 times. If we correct for the decreased range of the derived cycle because of the 2 hr sampling spread within each sample, i.e. divide by 0.72, the calculated maximum mitochondrial volume at the end of one division cycle would be 5.05 times the minimum. This rate of mitochondrial mass increase (5.05 times every 7.5 hr) would be equivalent to a mass doubling every 3.3 hr. Mycelial mass doubling for KJT1960a growing in culture on agar plates overlaid with dialysis membrane occurred every 3.4 hr (Fig. 1). This agreement between mycelial mass doubling time and mitochondrial mass doubling time is an indication that synchronous mitochondrial division is sufficient to maintain growth. Moreover, the close correspondence implies that synchronous

division is the only method of mitochondrial duplication.

Since the shape of some of the mitochondria does change during the cycle, however, the area-to-volume relationship expressed by equation 1 cannot be strictly true. The change in shape that is observed, from larger rounded mitochondria to elongated smaller mitochondria, would tend to make the observed range in volume larger than the actual range. This would cause the calculated, mitochondrial mass doubling time to move towards the mycelial mass doubling time. However, the change in shape is probably not sufficient to affect the results much, since most of the mitochondria observed are in neither the dumbbell nor the associated state (Table I).

A nonrandom process requires a mechanism of control. Sussman has evidence which shows that there is regulatory communication between *Neurospora* hyphae growing in the vicinity of each other, even though they are not anastomosed.[1] The mitochondrion has been found to contain DNA in significant amounts in *Neurospora crassa* (Luck and Reich, reference 12), and in numerous other plant and animal species. Mitochondrial DNA has been observed *in situ* (Nass and Nass, references 14, 15; Nass et al., reference 16; Schuster, reference 19) in many species, and Vogel (21) showed that DNA replication keeps pace with mitochondrial division. Furthermore, Reich and Luck (17) have presented positive evidence that the mitochondrial DNA of *Neurospora* has physical continuity through several cycles of mitochondrial replication, is metabolically independent of nuclear DNA replication systems to some extent, and is inherited in the same pattern as many abnormal mitochondrial phenotypes are inherited.

Some of the enzymatic components of the classical genetic system (DNA–RNA–protein) have also been demonstrated to occur in the mitochondrion. Luck and Reich (12) demonstrated the presence of RNA-polymerase activity in *Neurospora* mitochondria, and Wintersberger (26) found DNA–polymerase in yeast mitochondria. Clark-Walker and Linnane (4) demonstrated a protein-synthesizing system in yeast mitochondria. In addition to the evidence for the occurrence of the components of a genetic system, there is evidence indicating that this system is at work in the mitochondrion, specifying certain aspects of mitochondrial structure. Avers et al. (1) have shown in yeast the persistence of diverse mitochondrial genotypes and enzyme systems in common nucleocytoplasmic backgrounds, which is indicative of autonomous control of mitochondrial phenotype. This is strongly supported by the findings of Woodward and Munkres (27) who have demonstrated a mitochondrial protein alteration resulting from a cytoplasmic mutation, *pokey*, in *Neurospora*; and Monolou et al. (13) showed changes in the buoyant density of mitochondrial DNA in the cytoplasmic yeast "petite" mutants, which possess an altered mitochondrial protein.

It is proposed here that this mitochondrial genetic system is the focus of action of regulatory substances which act to control mitochondrial division, and that this division is in synchrony within a given area of the mycelial pad owing to intercellular regulatory communication. The regulatory substances may, of course, be under nuclear control.

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