FORMATION OF MITOCHONDRIA IN *NEUROSPORA CRASSA*

A Study Based on Mitochondrial Density Changes

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ABSTRACT

The choline concentration used in the growth medium influences the density of mitochondria produced by the *chol-1* mutant of *Neurospora*. Isopycnic centrifugation in sucrose gradients can be used to determine the density of mitochondria, and can resolve into two populations, mitochondria derived from a mixture of cells grown at low (1 μg/ml choline chloride) and high (10 μg/ml choline chloride) choline levels. In an experiment in which cells are shifted from low to high choline growth conditions, mitochondria obtained after varying time periods show a gradual decrease in density tending toward the level typical of high choline mitochondria. Over a 90-minute period of observation, during which time there is an increase of mitochondrial protein mass of ~ 50 per cent over that initially present, the mitochondria change density as a single population. These results are consistent with the view that mitochondria grow by random accretion of new lecithin into existing mitochondrial structures, and also that the mitochondrial population increases by division.

It has been concluded in an earlier publication (Luck, 1963 *a*, *b*) that "new" mitochondria are formed in growing cells by growth and division of preexisting mitochondria. The present study provides a further test of this conclusion. The test is based on the observation that the density of mitochondria produced by the *chol-1* mutant of *Neurospora* is determined, within certain limits, by the concentration of choline in the culture medium (Luck, 1965). Mitochondria produced at low choline levels (medium concentration 1 μg/ml choline chloride) are more dense than those produced at 10 μg/ml, and artificial mixtures of light and heavy mitochondria can be resolved into separate populations by isopycnic centrifugation in sucrose gradients.

A study of the density of mitochondria produced by an exponentially growing culture after a shift from low to high choline medium could provide a means of distinguishing between possible mechanisms of mitochondrial formation. If mitochondria grow and divide in a manner which randomly distributes newly added material throughout the old, it would be expected that, during the high choline growth period, the density of the entire mitochondrial population would gradually shift from a high density to the low density typical of mitochondria from cultures growing at 10 μg/ml choline. Persistence of a distinct population at low density would not be expected.

If mitochondria are formed *de-novo* from precursors of low molecular weight, two populations should be detectable in the above experiment. New mitochondria would appear at the characteristic low density without lag, while the high density of preexisting mitochondria would remain unchanged.

If mitochondria form from non-mitochondrial

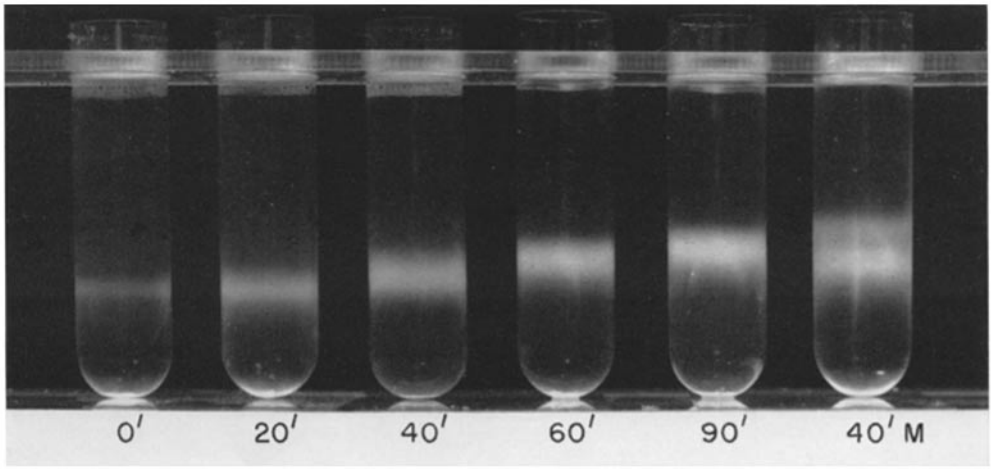

FIGURE 1 Isopycnic centrifugation of mitochondria obtained from *chol-1* cultures at various times after a shift from low to high choline growth conditions, as seen in a photograph of the centrifuge tubes.

Culture flasks containing 100 ml of minimal media and 1 μg/ml choline chloride were inoculated with 7×10^5 conidia per ml. After 15 hours' growth, choline chloride was added to a final concentration of 10 μg/ml, and at the times indicated a crude mitochondrial fraction was isolated, resuspended, and layered over a continuous sucrose gradient (1.9 M to 0.96 M, containing 0.001 M EDTA). The photograph shows the centrifuge tubes after 90 minutes centrifugation at 39,000 RPM in the SW 39 rotor of the Spinco model L ultracentrifuge (the corresponding centrifugal field is known to be adequate for mitochondria to attain density equilibrium).

Tube *40′M* shows the result of a mixing experiment designed to test the resolving power of the centrifugation method. The yield of a culture flask exposed to high choline for 40 minutes was mixed with $\sim$ 20 per cent its wet weight of a culture grown for 15 hours on high choline, and the mixed hyphal population was processed together. Since the doubling time of the mitochondrial protein mass is 150 minutes, during a 40-minute growth period a new mitochondrial population equivalent to about 20 per cent of that originally present would be expected. If that new population were produced *de-novo*, the density of the mitochondria should be that typical of continuous high choline growth. The experiment was designed to test the detectability of such a population even in the presence of a larger population of mitochondria the density of which might be altered. The satellite population is clearly visible in tube *40′M* (the result of the model experiment), but no such population is seen in any other tube.

membranous precursors, there should be no change in the band representing preexisting mitochondria. The number of mitochondria of intermediate density, and the delay in appearance of a distinct band at low density would be determined by the size of the precursor membrane pool. Experiments recorded in the preceding paper (Luck, 1965) indicate that if such a pool exists, it must be small. When *chol-1* cultures are shifted from low to high choline, there is no change in the cellular growth rate and the time course of radioactive choline uptake into mitochondria is linear. The time course of the dilution of choline-labeled mitochondria during growth in unlabeled high choline medium is also linear.

MATERIALS AND METHODS

The procedures used for growing *Neurospora*, isolating mitochondrial fractions from homogenized hyphae, and characterizing these fractions in terms of density, phospholipid/protein ratio, and enzymic activities are described in the preceding paper (Luck, 1965). Methods for electron microscopy can also be found there.

RESULTS

Changes in Mitochondrial Density

When choline is added to the medium of *Neurospora* cultures growing at a low concentration of choline, the density of the entire mitochondrial population decreases with time. Fig. 1 is a photo-

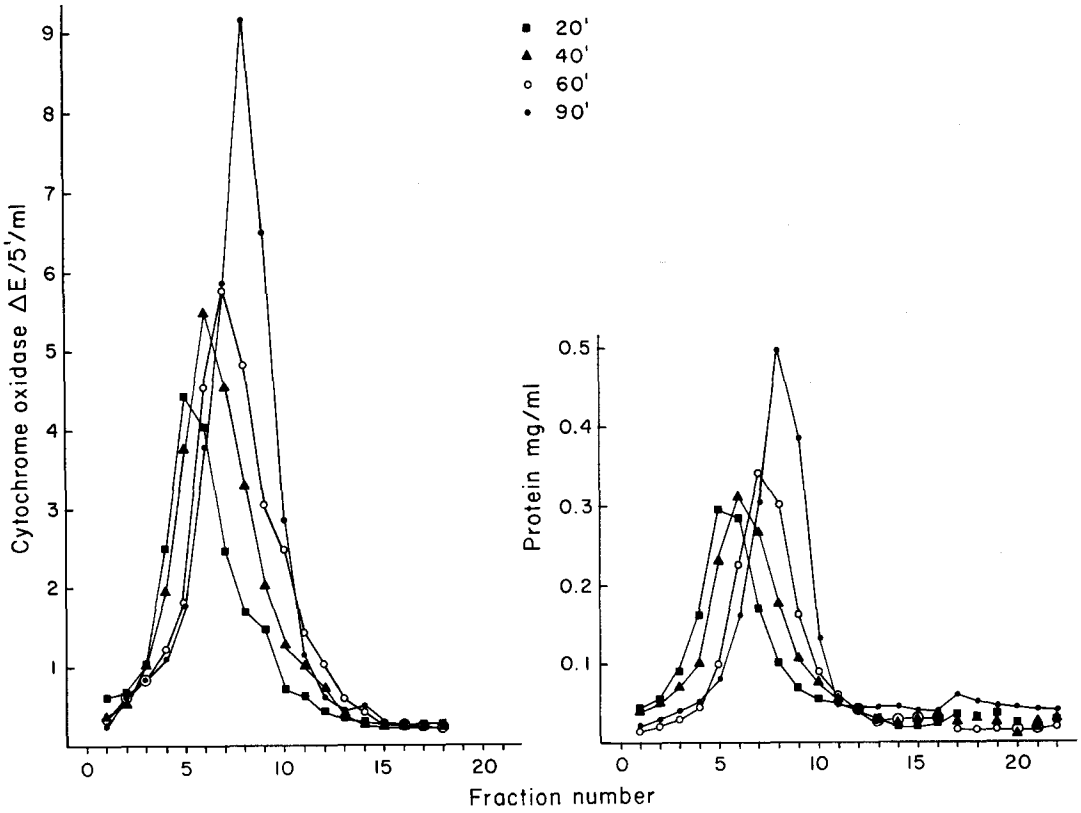

FIGURE 2 Distribution of cytochrome oxidase and protein in sucrose gradients after isopycnic centrifugation of mitochondrial fractions obtained from *chol-1* cultures, 20, 40, 60, and 90 minutes after a shift from low to high choline growth conditions.

Following isopycnic centrifugation in the experiment shown in Fig. 1, serial three-drop fractions were collected through a pin hole in the bottom of each centrifuge tube. Each fraction was collected in 1 ml 0.02 M tris buffer pH 7.4, with fraction 1 representing material at the bottom of the tube, and fraction 22 the top. Aliquots of the diluted material were assayed for cytochrome oxidase activity and for determination of total protein.

graph of the centrifuge tubes used for isopycnic sedimentation of mitochondria obtained from cultures at various times after addition of choline to the cultures. Throughout the 90-minute observation period mitochondria form a single band, and there is no suggestion of a satellite population at a density corresponding to that of mitochondria from high choline cultures (the upper band in the tube labeled *40'M* can be used as a reference for this density).

The distribution of cytochrome oxidase activity and total protein in serial fractions obtained from these gradients are shown in Fig. 2. These data identify the bands as mitochondria, provide a more quantitative estimate of mitochondrial distribution, and substantiate the conclusion that a gradual shift in density which affects the entire mitochondrial population occurs during the 90-minute period.

To test the resolving power of the method, we have carried out mixing experiments the results of which are shown in Fig. 1 (*40'M*) and Fig. 3. These experiments were designed to test whether a population of mitochondria produced *de-novo* during 40 minutes of growth in high choline medium could be detected even in the presence of a larger population of preexisting mitochondria with altered density. The model experiment (Figs. 1, 3) shows that the method could certainly identify such a population if it existed.

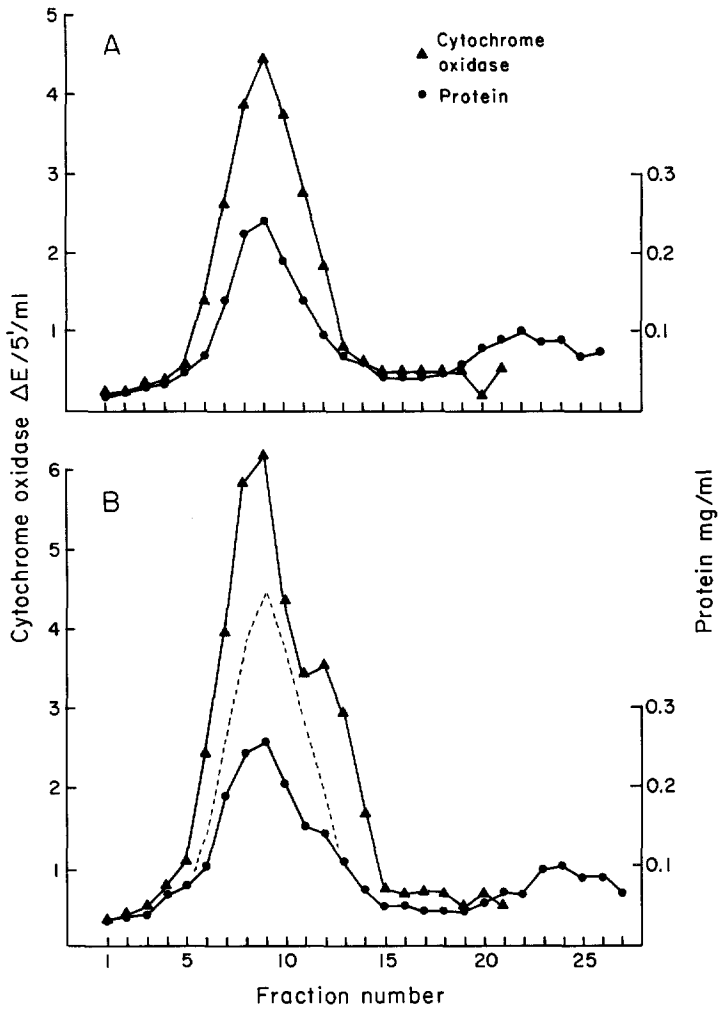

FIGURE 3 Distribution of cytochrome oxidase and protein in sucrose gradients after isopycnic centrifugation of mitochondrial fractions obtained from a mixing experiment.

FIGURE 3 *A* shows the distributions for a mitochondrial population obtained from a *chol-1* culture exposed for 40 minutes to high choline growth medium.

FIGURE 3 *B* shows the distributions for mitochondria derived from a mixture of a comparable culture of 40 minute cells with ~ 20 per cent their wet weight of cells grown continuously on high choline. The broken line indicates the distribution of cytochrome oxidase activity shown in Fig. 3 *A*.

The experiments are identical to those described in Fig. 1 (tube *40'* and *40'M*) and the method of collecting fractions from the centrifuge tubes is the same as that in Fig. 2.

Changes in Mitochondrial Morphology

The morphology of mitochondria is also influenced by the concentration of choline in the culture medium (Luck, 1965). Mitochondria of cells grown at 1 μg/ml choline are significantly larger than those of 10 μg/ml cells. It was, therefore, significant to find that electron microscopic studies of cultures made at various times following a shift from low to high choline growth conditions showed a gradual change in mitochondrial morphology which appeared to affect all mitochondria in a more or less synchronous fashion. Fifteen minutes after addition of choline to the culture medium (Fig. 4), the areas of the mitochondrial

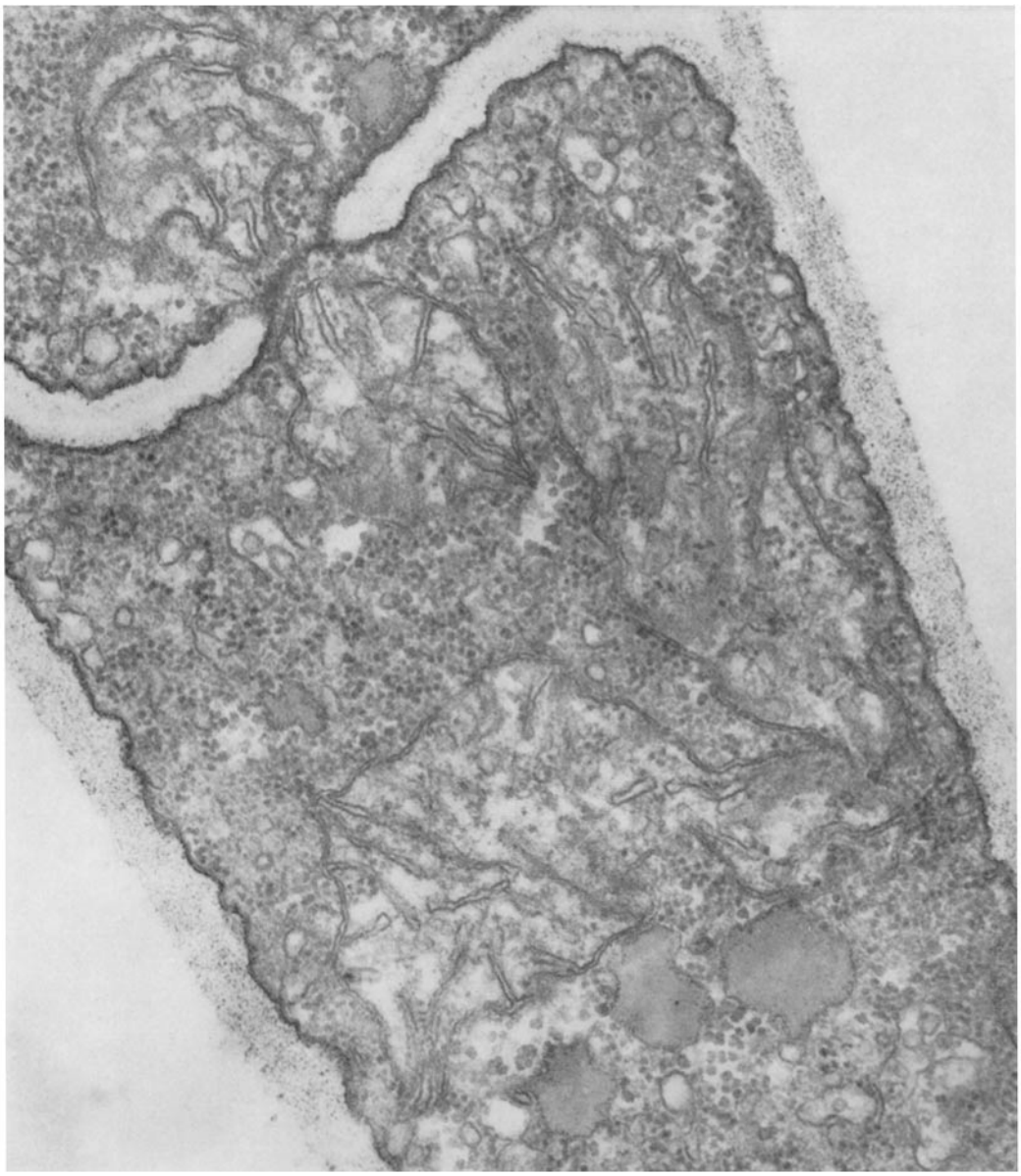

FIGURE 4 Electron micrograph showing a longitudinal section through a hyphal filament of the *chol-1* mutant of *Neurospora* 15 minutes after the level of choline chloride in the culture medium was increased from 1 μg/ml to 10 μg/ml. The change in choline concentration was made after the culture had grown 15 hours in the low choline medium.

The filament is divided by a typical incomplete septum with a mitochondrion in passage through the septal pore. The two mitochondrial profiles shown represent a low and median size, the usual diameters for mitochondria of this time point ranging from 0.45 to 0.65 μ. The presence of a constriction in the profile of the lower mitochondrion is another typical feature of this cell type. The figure should be compared with Figs. 4 and 6 *A* of the preceding paper (Luck, 1965). × 58,000.

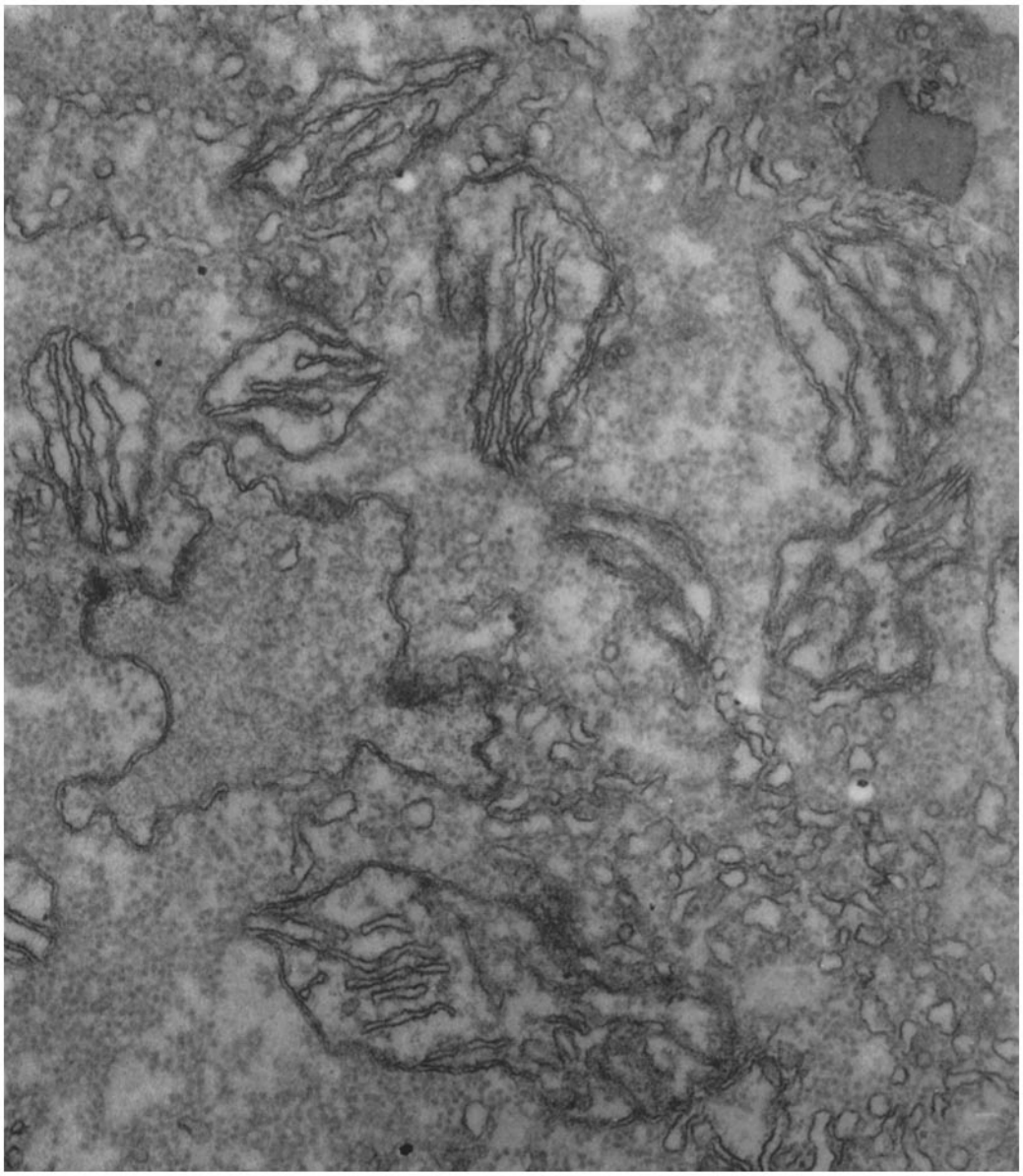

FIGURE 5 Electron micrograph showing a longitudinal section through part of a hyphal filament of the *chol-1* mutant of *Neurospora* 45 minutes after the level of choline chloride in the culture medium was increased from 1 to 10 μg/ml. The change in choline concentration was made after the culture had grown 15 hours in low choline medium. × 58,000.

The mitochondrial profiles seen in this section give a representative view of the size range typical of this cell type. At 45-minute time points, mitochondrial diameters ranged from 0.15 to 0.4 μ. The mitochondrial size distribution begins to resemble that encountered in cells grown for long periods on high choline (10 μg/ml choline chloride) medium (see Figs. 5 and 6 *B* of the preceding paper (Luck, 1965)).

profiles appear to be somewhat smaller than those of the initial low choline cells, and images showing constrictions in the mitochondrial outline are frequent. At 45 minutes (Fig. 5), the reduction in area of mitochondria is well advanced and the picture begins to resemble that of high choline cultures. Despite the fact that, at this time point, there is little more than a 20 per cent increase in the total mitochondrial protein mass, profiles with the appearance of typical "low choline mitochondria" are not seen. These results indicate that addition of choline to low choline cultures produces a change in mitochondrial morphology which affects all existing mitochondria. The kind of morphologic change encountered would be an expected result of the increased availability of phospholipids, if these materials were incorporated into additional outer membrane, dividing the mitochondrial volume into smaller units. It should be emphasized, however, that the outer membrane is not likely to be the only site for incorporation of the new phospholipid made available by increasing choline supplementation to the culture medium. Since the number of cristae per unit mitochondrial area does not substantially change during the transition, it is clear that some phospholipid is required for the formation of these structures. In addition, the decrease in mitochondrial dimensions appears to be nearly complete by 45 minutes, while the increase in phospholipid to protein ratio and the decrease in mitochondrial density continues for at least 90 minutes.

Preliminary observations made in living cells by light microscopy (for methods see Luck, 1963 *b*) are consistent with the suggestion that the increase in mitochondrial surface area is an early consequence of the increase in phospholipid content. Upon addition of choline, a progressive lobation of mitochondria is seen which increases the surface area to volume ratio. While it is clear that many of the arms formed by this process are resorbed back into the main mitochondrial mass, it is also apparent that pinching off of these lobes leads to an increase in the number of mitochondria. Since the conditions of culture which are required for observation with the light microscope are considerably different from those used in our experiments, we cannot yet safely conclude that lobation and "pinching off" are the basic mechanisms leading to change in the density and morphology of mitochondria.

DISCUSSION

The fact that the extent of choline supplementation to the culture medium influences the density of mitochondria produced by the *chol-1* mutant of *Neurospora* provides a means of testing hypotheses of mitochondrial formation. We have used isopycnic centrifugation in sucrose gradients to study the density characteristics of mitochondria obtained from cultures at various times after a shift from low to high choline growth conditions. The choline chloride concentration was raised from a level just adequate to support exponential growth at maximal rates (1 μg/ml) to a level which gave maximal choline incorporation into cellular phospholipids. During the 90-minute observation period, the mitochondrial protein mass increased by $\sim$ 50 per cent, and the mitochondria underwent a gradual decrease in density which affected the whole organelle population, as shown by the fact that at each step in the density shift the mitochondria formed a single band in the gradient. At no time was there a detectable population of mitochondria with the low density characteristic of high choline growth conditions. The resolving power of the centrifugation procedure used was adequate to detect such a population had it been present.

Electron microscopic studies of cells after the conversion to high choline growth conditions indicated that there were changes in mitochondrial morphology which could be satisfactorily correlated with the observed density changes. The large mitochondrial profiles typical of low choline cells were gradually replaced by smaller profiles so that, at 45 minutes, the mitochondria began to resemble those of high choline cells. The appearance of the mitochondria was fairly uniform for each time point, suggesting that the change affected all mitochondria.

These findings are not consistent with the hypotheses that mitochondria form during cell growth from non-mitochondrial membranous precursors or that there is *de-novo* formation. The most likely interpretation of the results is that the mitochondrial mass grows by accretion of new materials inserted randomly into the existing structure at a small dimensional scale, and that the mitochondrial population increases by division. These processes appear to proceed continuously in all existing mitochondria. The experimental findings do not give information concerning the

site of synthesis of the macromolecules required for increasing the mitochondrial mass, nor do they specify the precise way in which these components are added to the existing structure.

The conclusions reached in the present experiments are in precise agreement with those of an earlier radioautographic study (Luck, 1963 *a*, *b*). In both cases, the conclusions are based on observations of incorporation of lecithin into mitochondria of growing cells. The crucial finding is that "new" lecithin is introduced continuously and randomly into all existing mitochondria. In interpreting this result, consideration must be given to the possibility that the randomization observed might be a secondary phenomenon, involving a redistribution throughout the entire mitochondrial population of lecithin initially incorporated only in newly formed mitochondria. Possible mechanisms for such a redistribution, such as fusion and fission of mitochondria or randomization introduced by the cell fractionation procedure, have already been considered and rejected on the basis of experimental results (Luck, 1963 *b*).

It is possible, however, that redistribution of lecithin could be brought about by an efficient exchange mechanism operating at a molecular level. Although at present our results cannot unequivocally rule out such a mechanism, they impose a number of restrictions on such a process which make it seem unlikely. Since dilution experiments indicate that there is net conservation of lecithin in mitochondrial fractions during logarithmic growth, the molecular exchange process would have to be limited to mitochondria or could involve other cellular lecithin compartments only if these components showed growth and dilution kinetics similar to the mitochondria. (This question is further considered in the appendix.) In either case, the exchange process would have to be efficient enough to give equilibration in minutes among all lecithin compartments involved. An estimate based on the results of a 10-minute pulse labeling experiment (see Footnote 4, Luck, 1963 *b*) predicts that the exchange process would have to accomplish total equilibration of mitochondrial lecithin in $\sim$ 1.5 minutes. If the equilibration of lecithin into all cellular compartments is mediated by a non-membranous lecithin precursor pool, our results require that this pool be not significantly expanded by increasing the level of choline available to cultures. This is shown by the time course of uptake of radioactive choline and by the time course of dilution of specific activity in choline-labeled mitochondria during experiments in which cultures are shifted from low to high choline growth conditions.

If division coupled with growth by random accretion is the mechanism of mitochondria formation in exponentially growing *Neurospora*, can this mechanism be considered to hold for other cellular systems, and does it represent the only means of mitochondrial formation? The question is hard to answer because of the difficulty in designing critical experiments for other cellular systems. Studies based on interpretations of electron microscopic images (see reviews by Novikoff, 1961, and recent papers by Andre, 1962, and Bell and Mühlethaler, 1964) have provided support for the division hypothesis as well as for derivation of mitochondria from non-mitochondrial cellular structures; but the evidence in question cannot be considered as more than suggestive without further experimental studies. Two recent studies, one by Bahr and Zeitler (1962) demonstrating that isolated liver mitochondria can be classified into two groups, spherical and oblong in shape; and another by Brosemer, Vogell, and Bucher (1963) correlating the growth of mitochondrial membranes with the increase in mitochondrial enzymes in developing flight muscles of the locust, have been interpreted as evidence for growth and division of mitochondria.

Evidence for a derivation of mitochondria from non-mitochondrial membrane precursors has come from a study of anaerobic yeast. It has been claimed that the strictly anaerobic organism contains *no* mitochondria, but that these structures become apparent, along with a functioning respiratory system, after exposure to oxygen (Linnane *et al.*, 1962; Polakis *et al.*, 1963; Wallace and Linnane, 1964). The crucial feature in the interpretation of these experiments is the reliability of the observation that no mitochondrial structures are present in the initial anaerobic cells. Recently, Morpurgo *et al.* (1964) have published a study of *S. Cerevisiae* showing the presence of mitochondria in anaerobic cells (grown in the presence of ergosterol).

At present, our conclusions strictly apply to *Neurospora* in the logarithmic phase of growth. It is conceivable that they apply also for other aerobic cells in similar conditions. For cells in a stationary growth phase, like most cells of an adult metazoan,

the situation may be more complicated, and in organisms capable of transition from anaerobic to aerobic growth, mitochondrial formation may prove to be quite different.

APPENDIX

We have carried out additional experiments to test the possibility that lecithin is redistributed among mitochondria by a molecular exchange mechanism. (The experiments bear on a subject discussed on page 468). The experiments were carried out during a 10-minute pulse experiment (^{14}C choline), a time point at which it is known that radioactive choline is randomly distributed among the entire mitochondrial population (Luck, 1963 *b*); and they were designed to test whether or not the total cellular lecithin is involved in a molecular exchange.

Cultures (100 ml) growing for 15 hours under high (10 μg/ml) choline growth conditions were filtered and the mycelial mass transferred to similar flasks containing ^{14}C-methyl choline at the same concentration. After 10 minutes, the mycelia were collected by filtration and mitochondrial fractions were prepared in the usual way. From the supernate of the initial mitochondrial pellet a "membrane fraction" was prepared by centrifugation at 105,000 *g*(av) for 90 minutes. The pellet thus obtained was resuspended in 0.44 M sucrose, layered over a continuous gradient 0.58 to 1.9 M sucrose (all solutions were 1 mM in EDTA), and centrifuged in the SW 39 rotor of the Spinco preparative ultracentrifuge for 2 hours. Material forming multiple bands in the density range 1.08 to 1.14, which could be clearly distinguished from the faint residual mitochondrial band, was collected and pooled as a non-mitochondrial membrane fraction. This fraction had been shown in earlier experiments (Luck, 1963 *b*) to consist of membrane-bounded vesicles. Pooled mitochondrial (M, Table I) and pooled vesicular fractions (V) were precipitated with TCA, and lipids were extracted from the precipitates using the Folch (1957) method. Care was taken to wash the chloroform layer four times in order to eliminate any non-lecithin choline. The lipid extracts were hydrolyzed in 2 N H_2SO_4, and the choline content was determined using a *Neurospora* biological assay according to the method of Horowitz (1946). Radioactivity of the hydrolysate was measured in a scintillation counter.

The results (Table I) indicate that the specific radioactivity of lipid choline obtained from the V fraction is almost twice that of the mitochondrial fraction. An estimate of the relative choline content of the two fractions based on the yields recovered in these experiments indicate that the lecithin content of the vesicle fraction is about 1.5 to 2 times greater than that of the mitochondrial fraction.

These results indicate that, if a molecular exchange mechanism capable of redistributing mitochondrial lecithin operates, it must be segregated totally or in part from other cellular lecithin. The extensive protoplasmic streaming visible in the light microscope and the fine structure of *Neurospora* revealed by the electron microscope point to no cellular components or cytoplasmic organization which could provide the basis for such restricted molecular exchange.

TABLE I

Specific Radioactivity of Lipid Choline from Mitochondrial and Vesicular Fractions

	Experiment I	Experiment II
Choline specific activity CPM/μg choline chloride		
M fraction	1490	600
V fraction	2760	1190
Ratio V:M	1.85	1.98

It should be pointed out that the vesicle fraction is heterogeneous, and little is known concerning the kinetics of choline incorporation therein or the effect on incorporation of the choline level in the medium. It is, therefore, too early to attempt an explanation of the unequal labeling of the mitochondrial and vesicular fractions. It is not likely that the entire V fraction is a precursor of mitochondrial lecithin: the inequality in choline labeling is so large that a precursor-product relationship would be detectable in the kinetics of incorporation of dilution of mitochondrial choline.

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