FORMATION OF MITOCHONDRIA IN *NEUROSPORA CRASSA*

A Quantitative Radioautographic Study

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ABSTRACT

Cells of a choline-requiring mutant of *Neurospora crassa*, labeled with radioactive choline, were transferred to unlabeled medium. At various times during their subsequent logarithmic growth, a highly purified mitochondrial fraction was prepared by sucrose density gradient centrifugation, and the distribution of label among individual mitochondria was determined by quantitative autoradiography. Preliminary experiments indicated that, under the conditions of this "washout" experiment, choline served as a stable mitochondrial label. Radioautographic analysis showed that, in fully labeled mycelia and for three mass doubling cycles in the unlabeled medium, radioactivity was randomly distributed among all mitochondria; *i.e.*, the distribution of autographic grains among individual mitochondria followed a Poisson distribution. In experiments in which pulse labeling for 10 minutes was used, the label was randomly distributed among all mitochondria. The data suggest that the mitochondrial mass is increased by a continuous process of addition of new lecithin units to the already existing mitochondrial framework.

Although the question of mitochondrial origin has been a subject of speculation for many years, no experiment has provided a clear understanding of the mechanism by which these cell organs are formed. Observations with the light and electron microscopes (see reviews by Rouiller, 1960, and Novikoff, 1961) have suggested three possible mechanisms of formation: division of pre-existing mitochondria, *de novo* synthesis, and formation from other non-mitochondrial precursor structures. In the latter case, the plasma membrane, nuclear envelope, endoplasmic reticulum, and "microbodies," among other cell structures, were proposed as possible sources for mitochondrial membranes.

The experiments reported here attempt to distinguish between these basic possibilities. The general experimental scheme makes use of an isotopically labeled constituent of the mitochondrial membranes. Fully labeled cells are transferred to an unlabeled medium, and, at various time points in the subsequent logarithmic growth period, mitochondria are isolated by cell fractionation, and the distribution of label among individual mitochondria is determined by quantitative radioautography.

This procedure is an adaptation of a method developed and successfully applied to the study of the mechanisms of inheritance of labeled DNA in dividing bacterial cells (Forro, 1957, 1960; Painter, 1958; van Tubergen, 1961). As in these studies, the analysis of the distribution of autographic grains among the structures concerned (in our case, mitochondria) could be expected to fit one of three patterns: (*a*) A conservative distribution in which the proportion of labeled mitochondria during growth in unlabeled medium would decrease as a logarithmic

function of time, the average grain count per mitochondrion in the labeled population remaining the same. This type of distribution is consistent with *de novo* synthesis of mitochondria from unlabeled precursors. (*b*) A non-conservative distribution in which the label would be distributed among all mitochondria and the average grain count per mitochondrion would decrease as a regular function of cell growth. Such a pattern is consistent with mitochondrial multiplication by division. (*c*) An intermediate pattern, in which the label is not randomly distributed in the total mitochondrial population, and the proportion of labeled individuals would decrease less rapidly than in the truly conservative case and the total grain count of the labeled population might decrease, remain the same, or increase slightly. This pattern would be consistent with the formation of new mitochondria from labeled precursors, which on the basis of earlier speculation (see Novikoff, 1961) could be other cell membranes.

In our experiments we used choline as a label for mitochondrial membranes, because lecithin, in which it is incorporated, is a major constituent of the mitochondrial phospholipids (Biran, 1961; Getz, 1962), and because it is possible to obtain tritium-labeled choline with high specific radioactivity. The availability of a choline-requiring mutant of *Neurospora* provided an opportunity to achieve efficient and chemically specific labeling, and we chose the *chol-1* (No. 34486) mutant for study in these experiments. This mutant, first isolated by Horowitz and Beadle (1943), appears to be blocked at the earliest step in synthesis of choline from ethanolamine, and can make neither mono- nor dimethylethanolamine nor choline, though it can use the first two compounds for synthesizing choline (Horowitz, 1945, 1946).

RESULTS

Growth Conditions

The study outlined requires a uniform population of cells with a reproducible and prolonged logarithmic growth phase. Such growth conditions can be obtained with *Neurospora* by making use of shaken liquid cultures. When these cultures are started with a large conidial inoculum, the growth during the logarithmic phase is in the form of small clumps (1 mm), and, if shaking is vigorous, there is little tendency for formation of large mycelial mats or for adherence to the wall of the vessel.

Preliminary experiments with the *chol-1* (No. 34486) mutant indicated that the growth rate, based on dry mass measurements, did not change

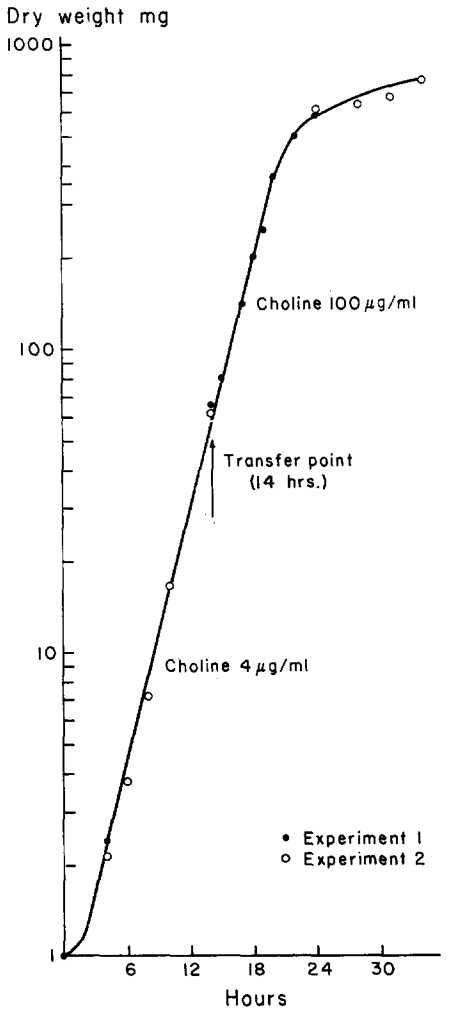


FIGURE 1

Dry mass increase in shaken cultures of *Neurospora crassa*.

300 ml Florence flasks containing 40 ml of minimal medium (Vogel, 1956) supplemented by 4 µg/ml choline chloride were inoculated with 7×10^5 conidia per ml. At 14 hours, contents of the flasks were transferred to 1000 ml Florence flasks containing 120 ml minimal medium with 100 µg/ml choline. The flasks, while maintained at a temperature of 27.5°C, were agitated in a reciprocating shaker at ~160 excursions per minute.

when choline chloride concentrations were varied from 1 to 100 μg/ml in shaken liquid cultures. This permitted us to use a two concentration growth system with an initial growth period at 4 μg/ml (total volume 40 ml) and a final period at 100 μg/ml (final volume 160 ml). In this system the initial period was used for labeling, and the later one for a 100-fold dilution of specific radioactivity of choline.[1] Fig. 1 shows the increase in dry mass in such a two-step growth system, and indicates that there is no alteration in the growth rate as a result of the transfer to a larger volume of medium and a higher choline concentration. During this unaltered logarithmic growth period the dry mass doubling time is 2 hours.

Zalokar (1959*a*) has pointed out that in shaken cultures, during the logarithmic growth period, hyphae grow mainly in length and show no cytological evidence of differentiation at different levels of the elongating filament. Though such filaments appear to be growing only at their tips, radioautographic studies (Zalokar, 1959*b*) reveal that protein and RNA synthesis proceed at the same rate throughout the entire filament. These observations suggest that, although *Neurospora* is a cenocytic fungus, in a shaken culture during log phase certain processes occur at a uniform rate along the entire filament.

The first requirement in our experimental scheme appears to be satisfied.

Preparation of a Mitochondrial Fraction

A highly purified mitochondrial fraction is required for the radioautographic analysis, because contamination by derivatives of other labeled intracellular membranes, not resolvable in the light microscope, could result in a high background. A very satisfactory mitochondrial preparation was obtained by isopycnic centrifugation in a continuous sucrose density gradient.

[1] An experiment described on page 494 indicates that at higher choline concentrations there is increased incorporation of choline from the medium into cellular phospholipids. The data indicate that there would be a 2.2-fold increased uptake of choline when the concentration in the medium is raised from 4 to 100 μg/ml. Therefore, with a 100-fold decrease in specific radioactivity at the time of dilution, the uptake of label by mitochondria would decrease to an insignificant level, at least by 45-fold.

The method for preparing such a fraction follows: 1 ml of homogenate, freed of nuclei and debris by a preliminary centrifugation (see Materials and Methods) and containing the equivalent of ~0.2 gm wet weight of mycelia, is layered over a 4 ml continuous sucrose gradient ranging in density from 1.24 to 1.08. Centrifugation is carried out in the SW 39 rotor of the Spinco model L ultracentrifuge at 40,000 RPM for 5 hours, and the centrifuge is stopped without braking. After centrifugation, the bottom of each tube is perforated and its content is collected into

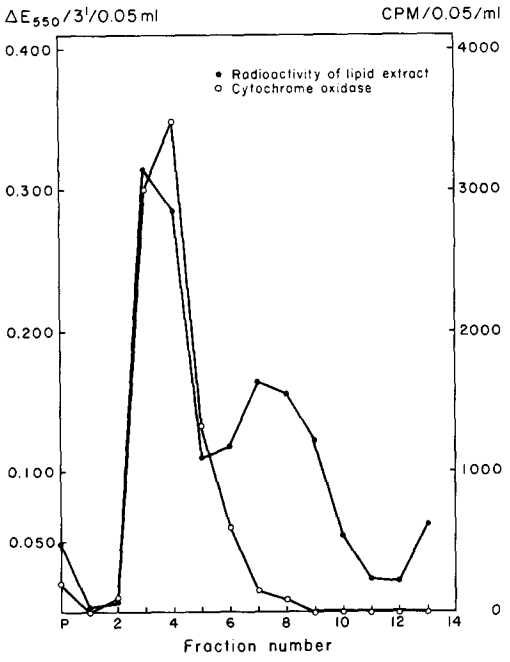


FIGURE 2

Distribution of cytochrome oxidase and lipid-extractable radioactivity in cell fractions prepared by centrifugation in a sucrose density gradient.

Preparation procedures are given in the text. Thirteen fractions, each containing the same number of drops, were collected through a pinhole in the base of the centrifuge tube. The small pellet (P) present was resuspended and also collected for analysis. Fraction 1 represents the material at the bottom of the gradient, and fractions 11, 12, and 13 represent the volume of the original sample load. Chemical and enzymatic analyses were carried out on aliquots of all fractions. The original homogenate was derived from cells grown for 14 hours in C^{14}-labeled choline (4 μg/ml) according to the procedure given in Fig. 1.

a series of fractions of known volume. Aliquots of these fractions are analyzed for cytochrome oxidase activity and for the lipid-extractable radioactivity of their trichloroacetic acid precipitates. The results obtained are illustrated in Fig. 2. Two peaks of radioactivity are found: one which coincides with an easily visible sharp band of material (fractions 3 and 4) at a density of 1.16 to 1.20, and another (fractions 7, 8, and 9) at a density of 1.08 to 1.14. The high density peak of lipid-extractable radioactivity coincides with the only peak of cytochrome oxidase activity and therefore appears to represent mitochondria. Neither the position of these peaks nor their general shape changes when centrifugation is prolonged to 15 hours.

The total cytochrome oxidase present in fractions 2 to 6 represents ~80 per cent of that present in the whole homogenate, whereas the lipid-extractable radioactivity of the same fractions accounts for 14 per cent of the total radioactivity extractable with lipid solvents from the whole homogenate.

Evidence that the high density band does consist of mitochondria, and an estimate of purity of the preparation were provided by electron microscopic studies. Fractions 3, 4, and 5 were fixed in suspension, centrifuged into pellets, and systematically examined with the electron microscope after embedding and sectioning. These pellets consisted almost exclusively of recognizable mitochondria like those shown in Fig. 3. The upper layer of each of the 3 pellets contained a thin layer of hollow circular profiles whose diameters were roughly in the same size range as those of the mitochondria. In each case the total depth of this layer was less than 10 per cent of that of the entire pellet. The appearance of the isolated mitochondria (Fig. 3) was compared with their appearance *in situ* (Fig. 4). Aside from the spherical form of the isolated organelles, the only marked change seems to be an over-all swelling and, in some cases, a ballooning of the outer membrane.

Electron microscopic study of the low density radioactive peak (fractions 7 and 8) reveals the presence of small, irregularly shaped, membrane-bounded vesicles. Recognizable mitochondria or mitochondrial fragments were very rare.

The enzymatic and electron microscopic studies already cited indicate that the visible high density band represents a highly purified mitochondrial fraction which, on the basis of cytochrome oxidase recovery, comprises ~60 per cent of the total mitochondrial population. The second prerequisite of our experimental scheme appears therefore to be satisfied. In subsequent experiments the mitochondria were routinely collected as an 0.8 ml fraction per tube, using as a guide the visible band already mentioned. All the radioactivity of this mitochondrial fraction is extractable by lipid solvents.

Stability of the Isotopic Label

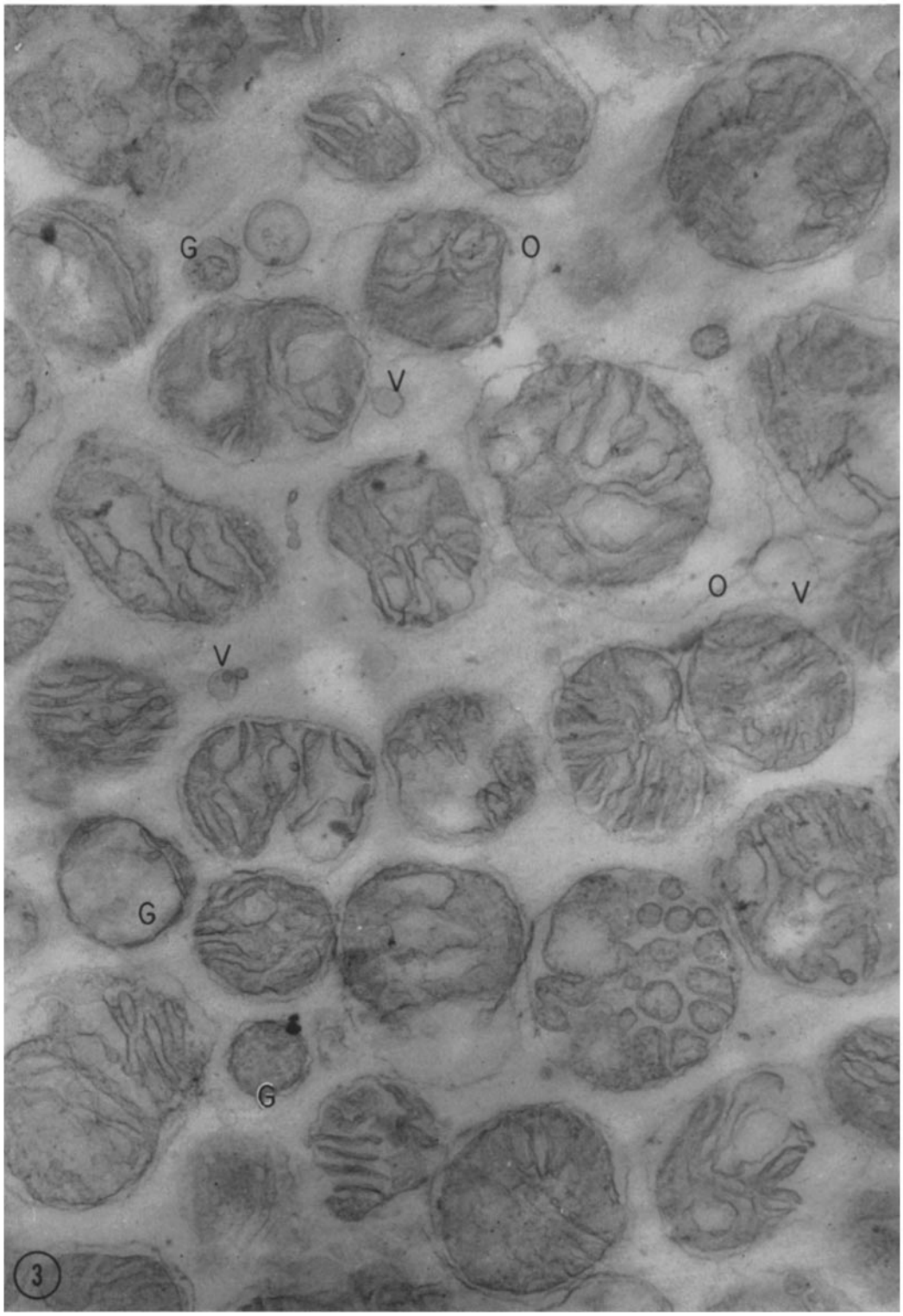
The study envisaged can succeed only if the radioactive choline behaves as a stable mitochondrial label. Hence, it is necessary to demonstrate that there is no metabolic turnover of the label, and that the radioactive choline is preserved in the mitochondrial population through three dry mass doubling cycles, in unlabeled medium.

To see if this requirement is satisfied, the following experiment was carried out: Isotopically labeled conidia were allowed to germinate and grow in labeled choline chloride at a concentration of 4 μg/ml, growth conditions being those given in Fig. 1. Choline used for the labeling of conidia and for their germination was of the same specific radioactivity. After 14 hours, the cultures were transferred to unlabeled media, and, at various time points during the subsequent 6 hours of logarithmic growth, individual flasks were har-

FIGURE 3

Electron micrograph of a representative field in a thin section from a mitochondrial fraction prepared by density gradient centrifugation.

The field shows many typical mitochondrial profiles with the usual pattern of cristae, as well as some profiles (*G*) which probably represent grazing sections of mitochondria. Many cristae are swollen and in some organelles the mitochondrial matrix appears fragmented. The outer membrane (*O*) shows relatively extensive blebbing. The few profiles of small vesicles (*V*) may represent sections through such blebs. × 34,000.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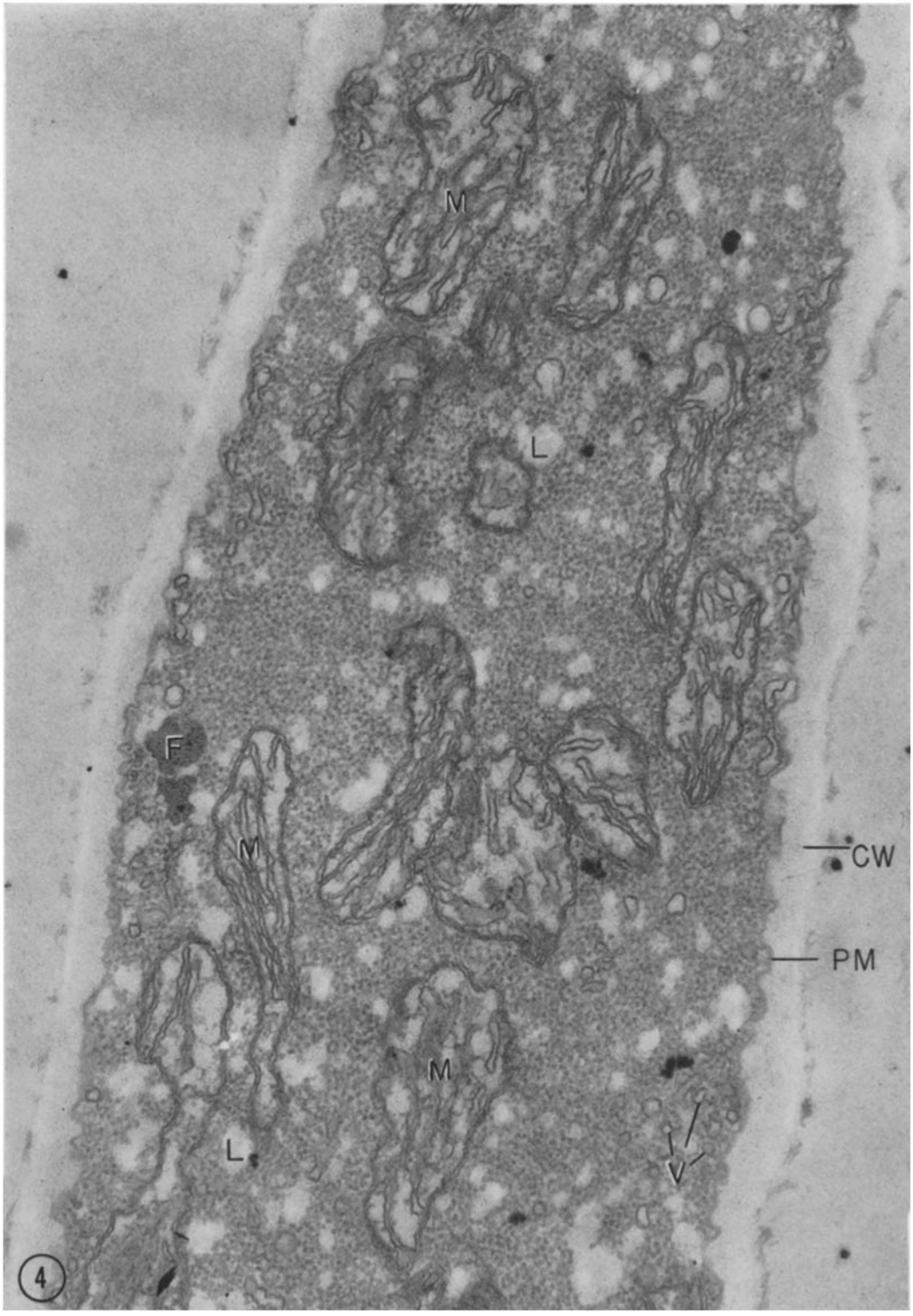
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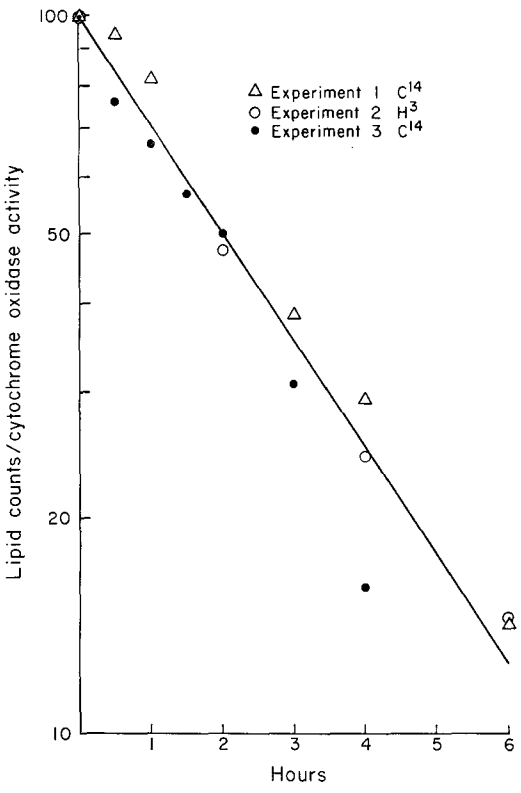

FIGURE 5

Decrease in "specific radioactivity" of mitochondrial fractions isolated during logarithmic growth of fully labeled mycelia in media supplemented with unlabeled choline.

Methods are given in the text. Results are plotted as lipid-soluble radioactivity (CPM/0.1 ml) per cytochrome oxidase activity (ΔE_{550}/3 minutes/0.1 ml) *versus* time of growth in unlabeled choline. Data from three separate experiments using H^3- and C^{14}-labeled choline are plotted on the same scale by taking the value at zero time in each experiment as 100, and expressing subsequent values as fractions thereof. The line is the theoretical line expected for a mitochondrial population (as measured by cytochrome oxidase), which increases with the dry mass and in which the choline label is preserved.

vested, the mycelia were homogenized and their mitochondria isolated by the procedure described. For each of the time points, lipid-soluble radioactivity and cytochrome oxidase activity of the mitochondrial fraction were determined. In Fig. 5, the results of three separate experiments are plotted, as the ratios of lipid-extractable radioactivity to cytochrome oxidase activity, against time of growth in unlabeled choline. The separate experiments have been fitted to the same scale by taking the value of this ratio at zero time as 100 and plotting the remaining values as fractions of that figure. The line is the expected decrease in this same ratio for a growing mitochondrial population in which there is no turnover of label and no pool, and whose doubling time is the same as that for dry mass doubling; *i.e.*, 2 hours. The experimental points fit closely this line.

The cytochrome oxidase activities recovered in the mitochondrial fractions during this 6 hour growth period are proportional to the wet weight of the mycelial mass. The average cytochrome oxidase activity, for the 17 observations shown in Fig. 5, is 920 ± 200 ΔE_{550}/hour/gm wet weight mycelia. The somewhat large random variation

FIGURE 4

Electron micrograph showing a longitudinal section through a hyphal filament of the *chol-1* mutant of *Neurospora* in a logarithmic growth phase (mycelia were grown for 14 hours in shaken liquid cultures).

The section shows numerous mitochondrial profiles (*M*) against a background of small, dense, ribosomal particles. In addition, the cytoplasm contains small vesicles (*V*), lipid inclusions (*F*), and irregular lacunae (*L*) which are probably occupied by polysaccharides. The cell is bounded by a plasma membrane (*PM*) and a cell wall (*CW*).

The change in mitochondrial morphology brought about by the fractionation procedure can be seen by comparing this section with Fig. 3. (It should be emphasized that the magnification here is 1.3 times that of Fig. 3.) Such a comparison shows that isolated mitochondria have undergone enspherulation, and significant swelling. The swelling is reflected in a distension of the space between inner and outer mitochondrial membranes, and as a dilatation of the intracristal space. Despite these changes, the basic structural elements (inner and outer membranes, and matrix) are preserved, and their normal topographic relationships generally maintained. × 44,000.

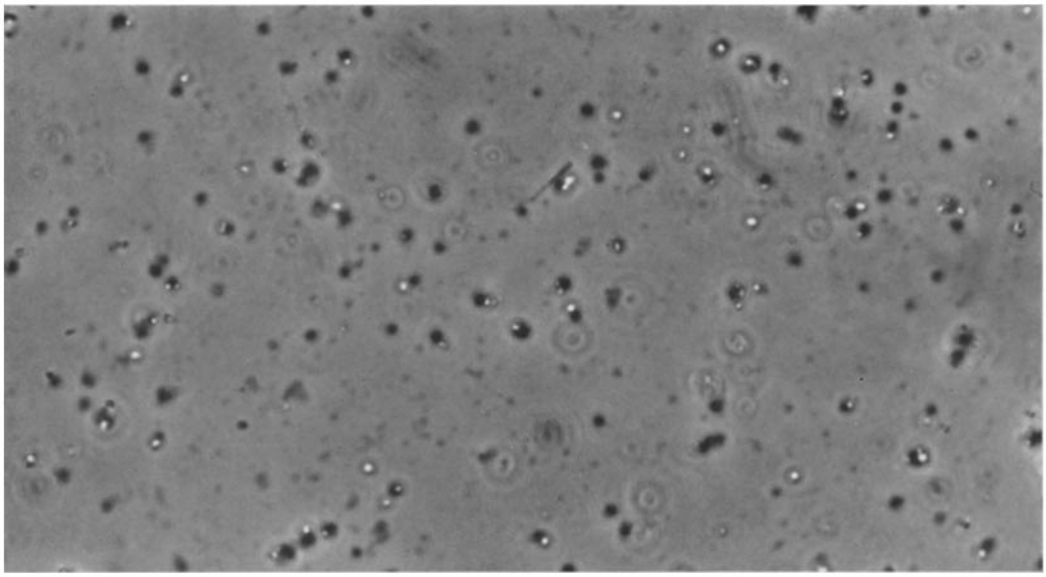

FIGURE 6

Phase contrast photomicrograph of a radioautograph of isolated mitochondria labeled with methyl-H^3-choline.

Photographic grains appear as bright spots in association with the larger, dark, round or oval mitochondrial images. There are few grains not associated with mitochondria, indicating that the background is satisfactorily low. Clumped mitochondria are not counted. The average grain count per mitochondrion for 300 mitochondria in this preparation is 1.3. × 2000.

of this value reflects variability in wet weight measurements, variable efficiency in mycelial homogenization, and differences in the collection of the mitochondrial fraction. None of these operations can be precisely controlled.

These results indicate that, during 6 hours of logarithmic growth in unlabeled choline, the mitochondrial population (as measured by cytochrome oxidase activity) increases as a function of time at a rate similar to the growth rate, and that the amount of isotopically labeled choline present at the beginning of the dilution period is preserved throughout three doublings of dry mass. No sizeable pool of radioactivity appears to contribute to mitochondrial labeling during this dilution interval. The requirement for a stable mitochondrial label is satisfied.

Radioautographic Studies

In these experiments conidia labeled with methyl-H^3-choline were germinated and allowed to grow over a period of 14 hours in a medium containing the same label at a concentration of 4 μg/ml. At that time, according to the procedure outlined in Fig. 1, the cultures were transferred to unlabeled media. At 2, 4, and 6 hours during the subsequent growth period, mycelia were harvested from individual culture flasks, and mitochondrial fractions were prepared, fixed in suspension, dialyzed, and finally spread on slides for radioautography. The fixation-dialysis procedure (see Materials and Methods) used preserved 90 to 100 per cent of the counts originally present

TABLE I

Average grain counts per mitochondrion during three dry mass doubling cycles of a labeled culture growing in unlabeled medium. The data presented are taken from one of two independent experiments in which the results show very close agreement.

Doublings	Exposure time for radioautograph			
	3 days	6 days	12 days	24 days
Zero time	2.0			
1τ	1.1	1.9		
2τ	0.5	1.1	1.8	
3τ	—	0.5	0.9	1.2

in the mitochondrial fraction. The appearance of the radioautographs after exposure and development is illustrated in Fig. 6. Under phase optics, when focus is at the level of the fixed and dried mitochondria, these organelles appear as dark round bodies and their associated photographic grains as bright spots of uniform diameter. By change of focus over each well separated mitochondrion, the total number of associated silver grains could be counted. Such counts were quite reproducible for any given specimen, for different observation times, and for different observers.

In Table I, the average grain counts for the three "doubling" times, obtained after different exposure times, are given. In general, these results conform to those predicted from the experiments of Fig. 5. It is to be noted that the average grain counts tend to fall below the predicted values with prolonged exposure times. This is especially true for 3τ at 24 days' exposure. At the shorter exposure times of 6 and 12 days, the value for 3τ is one-half that for 2τ. It is likely, therefore, that the discrepancy at 24 days' exposure is due to latent image fading (Demers, 1958).

A test of the randomness of distribution of label among mitochondria was made by applying the Poisson distribution function. If the mitochondria are uniform in the amount of label they contain, and if, on the basis of a large number of counts, the average number of grains per mitochondrion is $\bar{a}$, the probability ($P_{(i)}$) of finding i grains on a given mitochondrion would be expected to follow the relationship (van Tubergen, 1961).

$$P_{(i)} = \frac{\bar{a}^i\, e^{-\bar{a}}}{i!} \tag{1}$$

In order to provide enough categories of i to give a reliable test of fit to the Poisson equation, it is desirable to work with high values of $\bar{a}$. At high $\bar{a}$'s, however, some mitochondria are obscured by their associated grains. Therefore it was found useful to work at an experimental $\bar{a}$ of 2.0. To obtain such a value the exposure times were adjusted as follows: 3 days for zero time, 6 days for 1τ, 12 days for 2τ, and 24 days for 3τ.

In testing the fit of the data to the Poisson distribution, we made use of the linear plot suggested by van Tubergen and Setlow (1961). If equation (1) is rewritten in the form:

$$P_{(i)}i! = \bar{a}^i \cdot e^{-\bar{a}} \tag{2}$$

and the logarithm of both sides is taken, the following relationship is obtained:

$$\ln (P_{(i)} \cdot i!) = i \ln \bar{a} - \bar{a} \tag{3}$$

Therefore a plot of $\ln (P_{(i)} \cdot i!)$ *versus* i would give a straight line whose slope is $\ln \bar{a}$ and whose intercept is $-\bar{a}$ at $i = 0$. A straight line for any experimental $\bar{a}$ indicates a Poisson distribution. In Fig. 7, the results of our experiment are plotted in this manner. Each plot is based on a count of 600 grains, and for each division time the straight line represents the theoretical Poisson distribution for the value of $\bar{a}$ found experimentally. For all time points examined, there is good agreement between the experimental data and the Poisson distribution. For some time points there is deviation from the theoretical curve at high values of i. It should be pointed out that the value of $P_{(i)}$ for high i's is very low and therefore subject to great error. The deviation may also reflect the slight tendency for a single decay to produce a track in the emulsion (Caro, 1962).

The data presented in Fig. 7 indicate that, within the limitations of the method,[2] during logarithmic growth through three mass doubling cycles, the mitochondrial label is randomly distributed through the entire mitochondrial population. Fig. 5 and Table I indicate in addition that, during the same growth period in unlabeled medium, there is complete preservation of label in the mitochondrial population. The distribution of label appears to follow a non-conservative pattern consistent with the hypothesis that new mitochondria form by division of the old. Before this conclusion can be retained, other mechanisms for randomization must be considered and if possible ruled out.

Randomization of Mitochondrial Label Resulting From the Isolation Procedure

To check whether the homogenization and cell fractionation procedures introduce any randomization of mitochondrial label, a mixing

[2] Radioautographic analysis using low average grain counts (chosen for practical reasons) would not permit resolution of a mixture of labeled populations with small differences in their average grain counts.

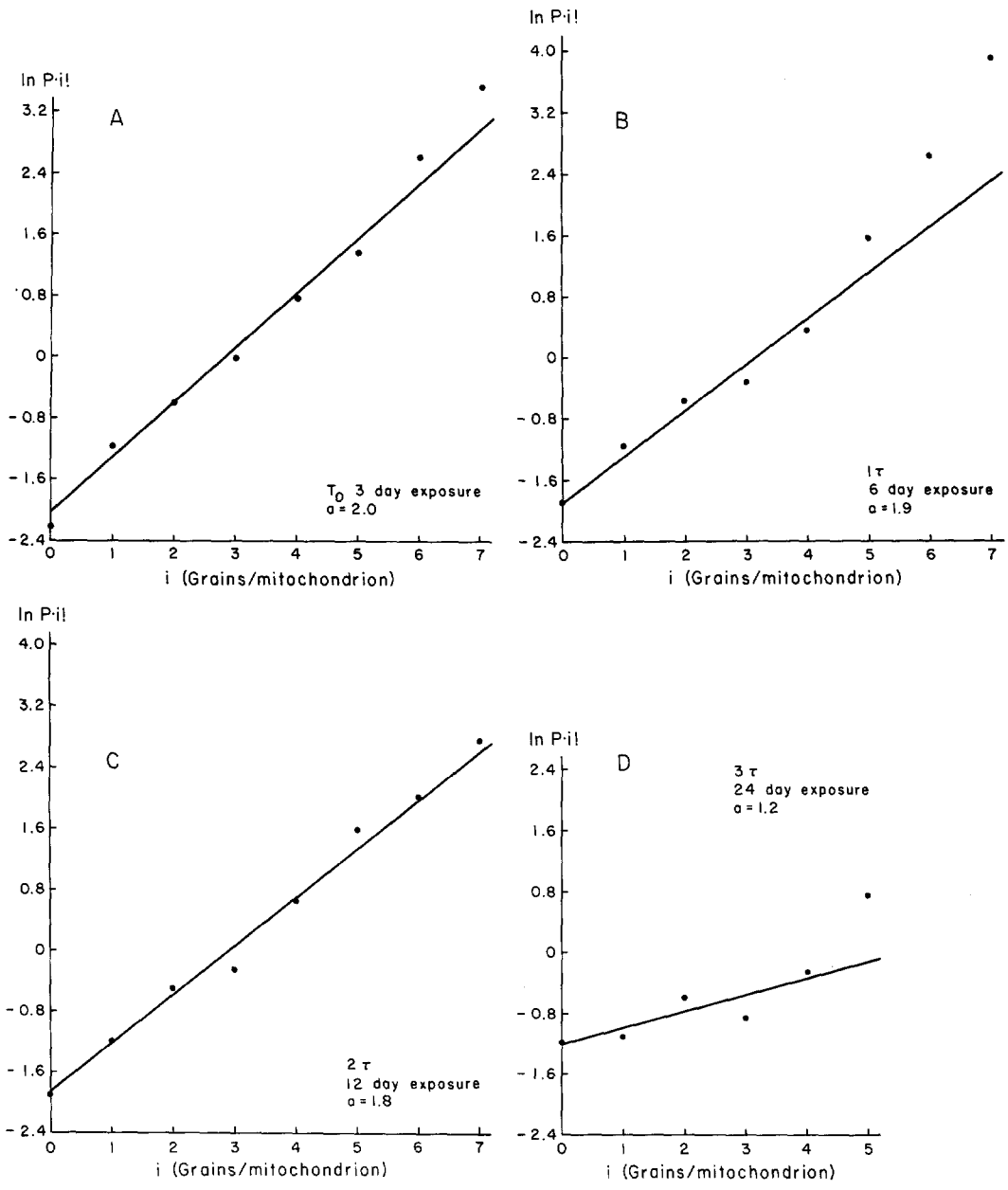

FIGURE 7

Distribution of radioautographic grains among mitochondria at zero time and at three "divisions" during growth in unlabeled medium. The data are taken from one of two independent experiments in which the results show very close agreement.

A. Fully labeled mitochondria (T_0). B. After 2 hours' growth in unlabeled choline (1τ). C. After 4 hours' growth in unlabeled choline (2τ). D. After 6 hours' growth in unlabeled choline (3τ).

Data are plotted as ln $(P_{(i)} \cdot i!)$ against i. For each time point the line represents the theoretical Poisson distribution expected for the experimentally determined value of $\bar{a}$.

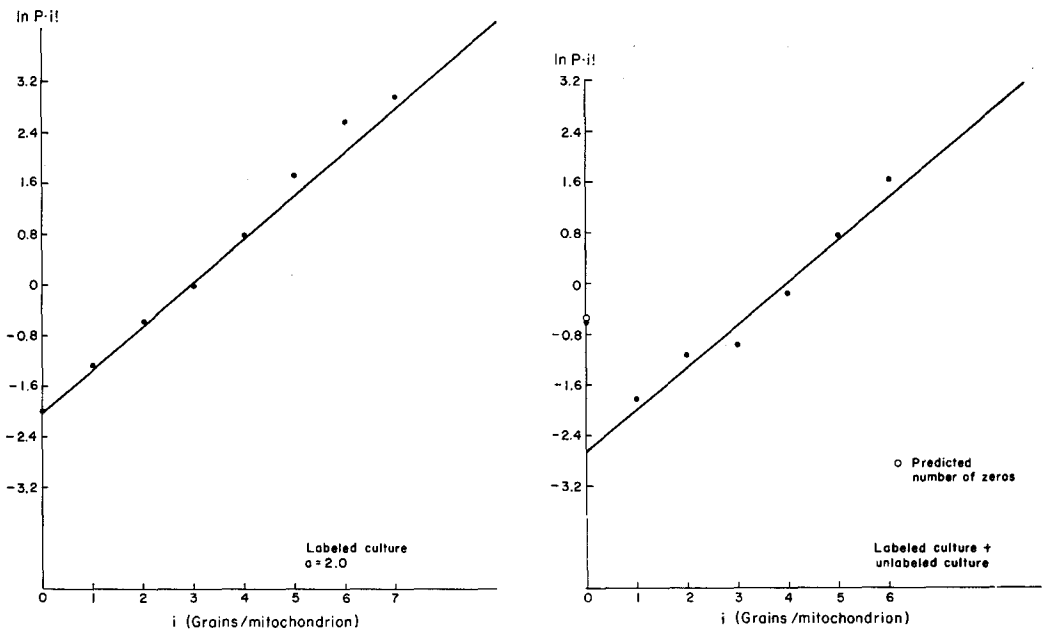

FIGURE 8

Distribution of grains over single mitochondria in a mixing experiment. The labeled culture shows a Poisson distribution of grains, with an average grain count of 2.0. The mixed culture fits closely the expected distribution for a mixed population of 50 per cent unlabeled mitochondria and 50 per cent labeled mitochondria with average grain count 2.0.* The data are taken from 1 of 2 independent experiments in which the results are in very close agreement.

* The predicted number of zeros and the line for the results of the mixed culture come from the following formulation:

In a mixed population where ϕ_l is the fraction of labeled units with average grain count $\bar{a}$, and ϕ_u the fraction of unlabeled units, in which $\phi_l + \phi_u = 1$, the probability of finding i grains on a single mitochondrion ($P_{(i)}$) is given by the following equation:

$$P_{(i)} = \phi_l \frac{e^{-\bar{a}}\,\bar{a}^i}{i!} + \phi_u(0)$$

at $i = 0$

$$P_{(o)} = \phi_l e^{-\bar{a}} + \phi_u$$

$$\ln P_{(o)} = \ln (\phi_l e^{-\bar{a}} + \phi_u) = \text{predicted number of zeros}$$

at other i's

$$P_{(i)} = \phi_l \frac{e^{-\bar{a}}\,\bar{a}^i}{i!}$$

$$\ln (P_{(i)} \cdot i!) = \ln \phi_l - \bar{a} + i \ln \bar{a}$$

when $\ln (P_{(i)} \cdot i!)$ is plotted against i it gives a line with slope $\ln \bar{a}$ and intercept at $i = o$ of $\ln \phi_l - \bar{a}$

experiment was carried out. Identical conidial inocula were placed in flasks containing 40 ml of media either with labeled choline chloride (4 μg/ml) or unlabeled choline chloride (4 μg/ml). At the end of 14 hours of growth, a labeled culture and an unlabeled culture were mixed and chilled. At the same time a labeled culture was harvested. Mitochondria were isolated and radioautographs prepared in the usual manner. Fig. 8 shows the distribution of grains over mitochondria in the labeled and in the mixed culture. The fully labeled culture shows an average grain count of 2.0 per mitochondrion, and a distribution of grains which obeys the Poisson relationship. The mixed culture can be resolved into a population with 50 per cent labeled cells, average grain count 2.0, and 50 per cent unlabeled cells. It follows that there has been no redistribution of the label during the grinding of the mycelia or during cell fractionation.

Randomization of Label by Mitochondrial Fusion and Fission

In their cinematographic studies of vertebrate cells in tissue culture, Frederic and Chèvremont (see Frederic, 1958) observed many changes in the form of mitochondria. One observation from these investigations, especially pertinent to our study, is that mitochondria apparently undergo repeated fission and fusion. A highly efficient process of fusion and fragmentation could account for the random distribution of label among all mitochondria. In order to investigate this possibility, we carried out a short term labeling experiment. If, at the end of a brief period of cell growth in radioactive choline, label is distributed among all mitochondria, we can obtain from the duration of the pulse an estimate of the frequency and efficiency of mitochondrial fusion that would be needed to account for complete randomization of the label. The possibility that such a process occurs at a sufficient rate could then be tested by microscopic examination of living *Neurospora*.

Shatkin (1960) found that the incorporation of inositol into the phospholipids of an inositol-requiring strain of *Neurospora* was increased when cells were grown with 4 times the inositol level required for maximal growth. If this were true for the *chol-1* mutant, it would provide a means of incorporating more label into mitochondria, and thus permit a short term labeling experiment for radioautography. In a preliminary experiment we found that the incorporation of radioactive choline into the lipids of the postnuclear supernatant increased at choline levels above those required for maximal growth rate, and reached a maximum when the choline chloride concentration of the medium was 10 μg/ml.[3]

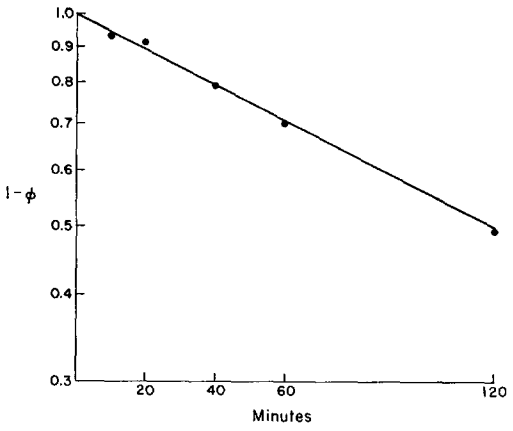

FIGURE 9

Time course of labeling of mitochondria during growth of cells in C^{14}-choline chloride (10 μg/ml).

After 14 hours of growth in 40 ml of medium (choline chloride concentration 10 μg/ml), cells were collected on Milipore filters (pore size 1.2 μ) and transferred to 80 ml of medium containing the same concentration of C^{14}-choline. At various time points during the subsequent growth period, culture flasks were chilled, their mycelia collected in Buchner funnels, and, after washing with 0.44 M sucrose, the cells were ground with sand. Mitochondrial fractions were isolated in the usual way, and determinations of lipid-extractable radioactivity and cytochrome oxidase activity were carried out.

Results were plotted as $1 - \phi$ against time, where ϕ is the ratio of specific activity (lipid-extractable count per cytochrome oxidase activity) at time (t) to the specific activity after a prolonged period of growth in label (the maximal specific activity). The time course of labeling should follow the following relationship (Britten, 1962): $1 - \phi = 2^{-\frac{t}{\tau}}$ where τ is the "doubling time." The line which is drawn is the theoretical line for $\tau = 120$ minutes.

[3] While in the case of the inositol-less mutant it seems clear that upon growth in high inositol there is a change in the composition of the phospholipids, in the case of choline the increased uptake of label may reflect a higher lecithin content of the phospholipids, or may be evidence that the *chol-1* mutant is "leaky."

The time course of labeling of mitochondria was studied by growing cells in unlabeled choline chloride (10 μg/ml) for an initial period of 14 hours, and then transferring them, after filtration, to media containing the same concentration of radioactive choline. The results of this experiment, which are shown in Fig. 9, indicated that the uptake of label into mitochondria fits closely

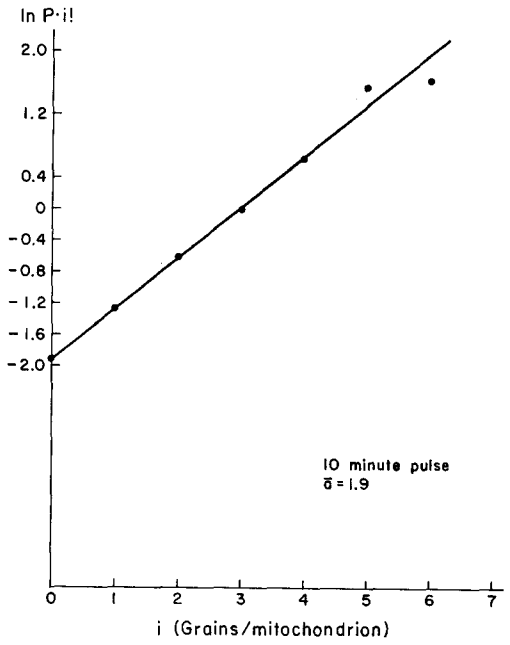

FIGURE 10

Distribution of radioautographic grains among mitochondria after 10 minute pulse labeling. Data are plotted as ln $(P_{(i)} \cdot i!)$ against i. The line represents the theoretical Poisson distribution for the experimental $\bar{a}$ of 1.9.

the theoretical line for a "doubling time" of 120 minutes. Thus, new choline was incorporated into mitochondria at a rate consistent with the dry mass doubling time, and the absence of a lag in the process suggested that there was no prolonged period in a pool.

These results indicated that a 10-minute pulse experiment for radioautography was feasible. Accordingly, conidia were grown in 40 ml of media containing 10 μg/ml choline chloride for 14 hours, and, after collection on a Milipore filter (pore size 1.2 μ), the mycelia were transferred to 40 ml of medium containing tritium-labeled choline at the same concentration. After 10 minutes, the culture flask was rapidly chilled and the mycelia were collected on a Buchner funnel, washed with 0.44 M sucrose, and ground with sand. Mitochondria were isolated and prepared for radioautography in the usual way, and after 21 days' exposure the distribution of autographic grains was determined. These results, which are shown in Fig. 10, fit the Poisson distribution and indicate that after 10 minutes, the label is distributed among all mitochondria.

Two models can be proposed to account for this distribution: the first assumes that labeled choline is added to the entire mitochondrial population in a continuous process; this model is consistent with the hypothesis that mitochondria multiply by division. The second model, consistent with the hypothesis of *de novo* origin, assumes that in the 10 minute pulse only newly formed mitochondria are labeled, and that randomization occurs by an efficient mechanism of fusion and fission.

An estimate of the frequency of such events, which would be required for labeling of the entire population, can be obtained by making certain limiting assumptions, namely, that upon fusion there is complete exchange of label between two mitochondria, and that each fusion is followed by fission. It can then be predicted that these events must occur per mitochondrion on the average of once per 1.5 minutes to give a population which is 80 per cent labeled.[4] This figure

[4] We are indebted to Bruce Knight of The Rockefeller Institute for the following derivation which is the basis of the estimation of the fusion-fission frequency required for randomization in 10 minutes:

α = the generation rate of mitochondria, defined so that the number of mitochondria (N) increases by a factor of e at time $1/\alpha$ minutes.

β = the fusion rate, the average number of mitochondrial fusions per minutes.

U = the number of unlabeled mitochondria.

$f = \frac{U}{N}$, the fraction of unlabeled mitochondria

t = time

The rate of decrease in the number of unlabeled mitochondria is given by:

$$\frac{dU}{dt} = -\beta \frac{N - U}{N} U = -\beta(1 - f)\, U \qquad (1)$$

the rate of formation of new mitochondria is given by:

represents a low estimate for the frequency of fusion-fission, because a 20 per cent unlabeled population could have been detected in the radioautographic analysis but was not, and because the assumption of complete interchange of label at fusion is probably unrealistic for a structure as complicated as the mitochondrion.

Visualization of mitochondria in living cells provided an opportunity to test the applicability of the second model. Small inocula of mycelia from shaken cultures were transferred to thin gelatin preparations (see Materials and Methods) and observed with Anoptral-contrast optics. In such preparations, cells of the *chol-1* mutant continued to grow, the growing tips advancing at 8 μ per minute. The filamentous or rod-like mitochondria could be identified, and small groups could be kept under observation for relatively long periods. Although mitochondria could most easily be observed at the growing tips, where they were not obscured by other cytoplasmic constituents, their behavior there was no different from that at other levels in the hyphae. Fusions between mitochondria were very rare, far less frequent than an average rate of 1 per 1.5 minutes per mitochondrion. Such fusions as were observed did resemble those recorded in the films of Frederic and Chèvremont,[5] in that the attachment was usually "head to tail"; there were, however, no further changes in shape of the fused pair which would reflect the internal rearrangements necessary for redistribution of labeled membranes. Fragmentations of mitochondria, which could be identified with more certainty than fusions, were extremely rare. Under observation, the mitochondria appeared to enlarge, and sometimes showed wave-like disturbances on their surfaces.

The observations of living *Neurospora* suggest that although fusions between mitochondria may take place, the process does not occur with enough regularity or at a rate fast enough to account for randomization of label in 10 minutes. This is also true for mitochondrial fragmentation which would have to occur at the same rate. Both in our observations and in the films of Frederic, fusion does not seem to be followed by the change in shape which would be expected to accompany the complete redistribution of label. For these reasons we would reject the second model as a satisfactory explanation for the experimental findings of the 10 minute pulse experiment. It seems more likely that the random distribution of label is the result of a continuous process of addition of labeled choline units to the entire mitochondrial population. The very low frequency of mitochondrial divisions found in living *Neurospora* is not inconsistent with this model, since the predicted rate of division would be no greater than 1 per 120 minutes.

DISCUSSION

In logarithmically growing cultures of *Neurospora crassa*, mitochondrial lipids can be labeled with H^3- or C^{14}-choline. This label is preserved through three mass doubling cycles when labeled cells are transferred to media containing unlabeled choline. During this period the specific radioactivity, expressed as lipid-extractable counts/cytochrome oxidase activity, decreases as a logarithmic function of time, following the expected course for a population with a doubling time of 120 minutes. Similar results are obtained in pulse-labeling experiments in which the time course of uptake of label by mitochondria fits the same doubling time. In both cases and within the limits of the time points chosen, there is no deviation in the labeling curves which would point to the existence of a sizeable, non-exchangeable precursor pool. These results indicate, therefore,

[5] A print of the film "Mitochondria" by Frederic and Chèvremont was obtained from the Tissue Culture Association, Duke University School of Medicine, Durham, North Carolina.

(*continued from preceding page*)

$$\frac{dN}{dt} = \alpha N, \text{ and } \frac{d}{dt}\frac{1}{N} = -\alpha\frac{1}{N} \qquad (2)$$

the rate of decrease of the fraction of unlabeled mitochondria is given by:

$$\frac{df}{dt} = \frac{d}{dt}\frac{U}{N} = \frac{1}{N}\frac{dU}{dt} + U\frac{d}{dt}\frac{1}{N}$$

Substituting from (1) and (2) above

$$\frac{df}{dt} = -\beta(1-f)f - \alpha f$$

which upon integration gives:

$$\frac{1}{f_{(t)}} = \frac{\alpha}{1+\frac{\alpha}{\beta}}e^{(\alpha+\beta)t} + \frac{1}{1+\frac{\alpha}{\beta}}$$

that choline can serve as a satisfactory mitochondrial label.

Radioautographic analyses show that this label is randomly distributed within mitochondrial populations, isolated as mitochondrial fractions from fully labeled cells as well as from cells undergoing logarithmic growth through three subsequent mass doubling cycles in unlabeled choline. This random distribution of radioactivity is not a result of the procedures involved in the isolation of mitochondrial fractions nor does it seem likely to result from a redistribution of label carried out by an efficient process of mitochondrial fission and fusion *in vivo* (as indicated by the data obtained in a 10 minute pulse experiment).

At the end of each doubling cycle the average count of autographic grains per mitochondrion is one-half that found at the end of the preceding cycle. Since the mitochondrial label is retained, and its distribution is random, the average grain counts suggest that the mitochondrial population (as represented by mitochondrial fractions) is also doubled.

These data, which fit the non-conservative model discussed previously, exclude the possibility of *de novo* formation of mitochondria and do not favor the hypothesis of structural mitochondrial precursors. The most likely interpretation of the findings is that in growing *Neurospora* the mitochondrial mass is increased by a continuous process of addition of new choline-containing lipid units to the existing structural framework. The number of individuals in the population is increased by division of mitochondria. The division process distributes the label at random, so that pre-existing mitochondrial membranous material is transmitted uniformly or nearly uniformly to all progeny. This indicates that the insertion of new material takes place at a level considerably below that of the mitochondrion. The data do not indicate whether the new material is inserted as phospholipid molecules newly synthesized within the mitochondrion, or as phospholipid-protein units formed outside the mitochondrion.

While multiplication of mitochondria by division seems well established it should be pointed out that these and other findings indicate that there are important distinctions between the process of mitochondrial division and the replication division process involved in the formation of chromosomes. In the time scale of 10 minutes ($\frac{1}{12}$ of a division cycle) the insertion of new membrane material appears to be a continuous, noncyclic process. For 3 generations at least, there appears to be no large scale, ordered growth sequence; old lipid units are uniformly represented in progeny mitochondria. Light microscopic observations *in vivo* (Frederic, 1958) indicate that there is variability and change in the size and form of mitochondria of a single cell. These observations suggest that mitochondrial division is less rigorously controlled than other cellular division processes. This interpretation is consistent with the view that the mitochondrion is an aggregate of equivalent functional units.

MATERIALS AND METHODS

Materials

Cytochrome *c*, type III (horse heart) was purchased from Sigma Chemical Company, St. Louis, Missouri. C^{14} and H^3 methyl-labeled choline chloride were purchased from the New England Nuclear Corporation, Boston. The specific activity of the tritium-labeled compound used in the radioautographic experiments was 100 mc/mMole.

The *chol-1* mutant, No. 34486, of *Neurospora crassa* was obtained from the Fungal Genetics Stock Center, Dartmouth College, Hanover, New Hampshire.

Culture of Neurospora

Stock cultures were maintained at 25°C on slants of complete media (Lester, 1959; Tatum, 1950) containing 1.5 per cent agar.

Large-scale production of conidia was carried out in 125 ml Erlenmeyer flasks containing 15 ml of minimal medium (Vogel, 1956) supplemented with 25 μg choline chloride/ml and 2 per cent agar (a thin coating of the medium covered the walls of the flasks). Small conidial inocula from the stock cultures were allowed to germinate and grow in such flasks at 30°C for 3 days. At this time, following the suggestion of Zalokar (1959a), flasks were moved to a 25°C room and were illuminated with a 60 watt incandescent lamp at a distance of 14 inches. After 2 days, conidia were harvested with a loop into a small volume of minimal media; they were freed from their mycelial attachments by shaking with glass beads, and separated from mycelia by filtration through a thin layer of glass wool. Aliquots of the filtered conidial suspension were counted in a Spencer "bright-line" counting chamber, and shaken culture flasks were routinely inoculated to give a final conidial concentration of 7×10^5/ml.

Preparation of Homogenates

Contents of culture flasks were collected on a Buchner funnel, washed with 0.44 M sucrose (containing 0.001 M EDTA), and then ground in a mortar with one-half equivalent wet weight of acid-washed Berkshire sand (Fischer Scientific Company) in 2.5 volumes of cold 0.44 M sucrose (0.001 M EDTA). Grinding was continued until the preparation became a smooth paste, and then the material was transferred to a 12 ml conical glass centrifuge tube with an additional 2.5 volumes of sucrose solution. A preliminary centrifugation to remove nuclei and debris was carried out in the International Centrifuge at 500 *g* average for 10 minutes. The final volume of the supernate was adjusted to give the equivalent of 0.2 gm wet mass per ml. All operations were carried out at 4°C.

Preparation of Continuous Sucrose Density Gradients

Linear 4 ml gradients were made using the method of Britten and Roberts (1960); 1.9 and 0.58 M sucrose (both containing 0.001 M EDTA) were used to load the chambers of the apparatus. Preliminary experiments with methyl orange as a marker for the 0.58 M sucrose indicated that the gradient was linear, and that it was stable for at least 24 hours. Gradients were routinely prepared at 4°C the night before an experiment.

Analytical Procedures

Lipid-soluble radioactivity was determined on aliquots of fractions after an initial trichloroacetic acid precipitation (10 per cent). After one washing with 5 per cent TCA, the precipitate was extracted for 30 minutes at room temperature with 2:1 chloroform: methanol (Folch, 1957), and, after centrifugation, samples of the supernate were taken for counting. Radioactive counts were measured either in a Nuclear Chicago model 181 A counter, equipped with a micromil window, or in the Packard tricarb liquid scintillation counter.

Cytochrome oxidase was assayed according to the method of Nielsen and Lehninger (1955) using a Beckman model DU spectrophotometer equipped with a Gilford optical density converter and automatic sample changer. Cytochrome *c* was reduced with sodium borohydride (Martin, 1958). When assays were carried out on samples from the sucrose gradient, maximal enzymatic activities were obtained only when such samples had been diluted 1:3 in 0.02 M Tris buffer (pH 7.4) before addition to the assay tubes. When this dilution step had been carried out, no stimulation of activity was observed when deoxycholate was added to the assay system. Enzymatic activities were calculated from the initial zero order rates.

Radioautography

Mitochondrial preparations (0.8 ml samples from the sucrose gradient) were fixed by addition of 0.2 ml of 4 per cent osmium tetroxide, and after 45 minutes were transferred to Visking bags and dialyzed overnight against water at 4°C. Uniform droplets of the suspension were transferred with a 5 mm platinum loop to microscope slides which had been previously coated with a 0.2 per cent gelatin–0.01 per cent chrome alum mixture. The slides were dried at 45°C.

Coating of the slides with emulsion, storage, and development were carried out according to the methods of Caro and van Tubergen (1962) except that Ilford L-4 nuclear emulsion was used instead of the K-5 emulsion specified in the original procedure.

Microscopic Observations

Growing hyphae of *Neurospora* were observed under Anoptral-contrast optics using a method of specimen preparation suggested by Dr. C. F. Robinow. Small inocula were placed on a thin layer of ~15 per cent gelatin in choline–minimal medium and sealed under a coverglass with trapped air spaces. In such preparations, at ~26°C, the growing tips of the filaments advanced at the rate of ~8 μ per minute.

Intact mycelia were fixed for electron microscopy using the method of Kellenberger, Ryter, and Séchaud (1958). Mitochondrial fractions were fixed in suspension with 1 per cent osmium tetroxide, and after 45 minutes were diluted with water and centrifuged into a pellet. The mitochondrial pellet was cut and oriented in the embedding material according to the method of Palade and Siekevitz (1956), to permit systematic examination of the entire depth of the fraction. Specimens were embedded in Epon (Luft, 1961) and sectioned with the Porter-Blum microtome. After staining with lead (Karnovsky, 1961) and sandwiching with a thin layer of carbon, sections were examined in the RCA EMU-2B and EMU-3F microscopes.

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