THE INFLUENCE OF PRECURSOR POOL SIZE ON MITOCHONDRIAL COMPOSITION IN *NEUROSPORA CRASSA*

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ABSTRACT

The chemical composition of mitochondria obtained from exponentially growing *Neurospora* can be varied by addition of choline or amino acids to the culture medium. The variation affects the phospholipid to protein ratio, and the density of mitochondria as determined by isopycnic centrifugation in sucrose gradients. These variations have been observed in biochemical mutant strains as well as wild type cultures. In a choline-requiring strain, two levels of choline supplementation to the medium have been defined: a low choline concentration just adequate to support maximal logarithmic growth, and a high choline concentration which permits maximal incorporation of radioactive choline into cellular lipids. Mitochondria isolated from cultures growing at the low choline concentration have one-half the phospholipid to protein ratio of those from high choline cultures, and their density is significantly higher. Artificial mixtures of the two types of mitochondria can be resolved into two populations by isopycnic centrifugation. The concentration of cytochromes (measured by mitochondrial difference spectra) and of malate and succinate dehydrogenases (measured by enzyme activity) were the same in both types of mitochondria, on a protein basis. The results suggest that during growth of the mitochondrial mass, the incorporation of phospholipid and protein components can vary independently. Direct kinetic measurements did indeed show that choline, added to a culture growing at low choline concentration, was incorporated into mitochondrial lipids at a rate faster than the incorporation of protein. The mitochondrial phospholipid to protein ratio can also be influenced by the level of leucine supplementation to a leucine-requiring mutant, so that with leucine concentrations above those required for maximal exponential growth, mitochondria of increasing density and decreasing phospholipid to protein ratio are produced. Additions of choline or amino acids to the minimal medium of wild type cultures influence mitochondrial composition in a manner directly comparable to that observed in biochemical mutant strains. The results suggest that mitochondrial composition, in general, is determined by rates of incorporation of the two major components, phospholipid and protein; that these rates can vary independently in response to precursor concentration in the culture medium; and that they normally operate at a precursor (substrate) concentration below saturation level.

Recently published observations (Luck, 1963 *a*, *b*) indicate that *Neurospora crassa* is a well suited organism for studying mitochondrial formation in growing cells. When grown in liquid culture, the fungus has a reproducible and prolonged logarithmic growth phase with a mass doubling time of about 2 hours, and its mitochondria can be isolated as highly purified fractions by using

isopycnic centrifugation in sucrose gradients. The kinetics of the incorporation of radioactive choline[1] into mitochondrial fractions isolated from a cholineless mutant have shown that the mitochondrial mass increases at the same rate as the cell mass in exponentially growing cells. Tritiated choline incorporated into mitochondria is retained when a large excess of unlabeled choline is added to the cultures, and quantitative radioautography shows that during growth in such a washout period the label is distributed uniformly among individual mitochondria. The finding suggests that the mitochondrial mass grows through the accretion of new materials to existing mitochondrial structures, while the mitochondrial population increases by division.

The availability of choline-deficient and other biochemical mutant strains not only provides opportunities for chemically specific labeling of mitochondria, but also makes possible an inquiry into the constancy of mitochondrial chemical composition and the extent to which it can be influenced by the available concentration of required precursors. The present study is concerned with such problems. Its results indicate that the mitochondrial content of lipid and protein is not fixed, and can be altered by the level of available precursor substances; these alterations can occur without change in the cellular growth rate, and can be observed in mutant strains supplemented with varying levels of the required compounds, as well as in a wild type strain when choline or amino acids are added to the medium. Kinetic studies indicate that rates of lipid and protein incorporation into mitochondria may vary independently, and that the rates reflect the concentration of available precursors. Alterations in chemical composition are associated with changes in mitochondrial structure.

MATERIALS AND METHODS

Neurospora Strains

The following strains of *Neurospora crassa* were used:
1. *Chol-1* (34486), a choline-requiring strain, blocked at the first step of methylation of ethanolamine to form choline (Horowitz, 1945, 1946).
2. *Em* 5256, a wild type strain isolated by Emerson and Cushing (1946).
3. *Leu-1* (D 221), a strain deficient in α-hydroxy-β-carboxyisocaproate dehydrogenase activity (Gross, 1963).
4. *Chol-1, Leu-1*, a double mutant obtained as a single ascospore from a cross between 34486 and D 221.

Strains 34486 and *Em* 5256 were obtained from the Fungal Genetics Stock Center, Dartmouth College, Hanover, New Hampshire. D 221 was obtained from Dr. S. R. Gross, Duke University, Durham, North Carolina.

Culture of Neurospora

In all experiments, *Neurospora* was grown in shaken liquid culture starting from a standard conidial inoculum (7×10^5/ml). Methods of preparing conidia and culture media used can be found in an earlier publication (Luck, 1963 *b*). In the present series of experiments, the shaken liquid cultures were maintained at 25° instead of 27°C used previously. At this lower temperature, the doubling time of the culture was $\sim$ 150 minutes.

Preparation of Mitochondria

The preparation of homogenates and sedimentation of nuclei and cell debris are described in an earlier publication (Luck, 1963 *b*). In the present series of experiments, homogenates, freed of nuclei and debris, were centrifuged at 14,000 RPM in the 40.3 rotor of the Spinco model L preparative ultracentrifuge (tubes were filled to capacity $\sim$ 6 ml) for 20 minutes to yield crude mitochondrial pellets which contained about 80 per cent of the total cytochrome oxidase of the nuclear supernate. Each pellet, the total yield from 50 to 100 ml of cultures, was resuspended in 0.5 to 1.0 ml 0.44 M sucrose (0.001 M EDTA) and layered over a 4-ml continuous gradient (Britten, 1960) ranging from 1.9 to 0.96 M sucrose (0.001 M EDTA). When such a gradient was centrifuged for 90 minutes at 39,000 RPM in the SW 39 rotor, the mitochondria separated as a single distinct band, which could be collected through a perforation in the bottom of the centrifuge tube.

Analytic Procedures

Cytochrome oxidase assays and determinations of lipid-soluble radioactivity were carried out by the methods used in our earlier work (Luck, 1963 *b*). Phospholipid-phosphorus determinations were made on aliquots of mitochondrial fractions after an initial trichloroacetic acid (TCA) precipitation (10 per cent). Lipids were extracted according to the methods of Folch (1957), using 0.04 per cent calcium

[1] Radioactive choline is incorporated into mitochondrial lecithin. When *chol-1* cultures are grown on methyl-^{14}C-choline, the total mitochondrial radioactivity is recovered in the lipid extract. Analysis of the lipid extract using silica-gel thin layer chromatography indicates that more than 90 per cent of the counts move with the R_f of lecithin.

chloride to wash the crude chloroform-methanol extract. Total phosphate of the washed lower phase was determined by Bartlet's (1959) procedure.

Protein was determined on TCA precipitates by Lowry's (1951) method, using bovine serum albumin as a standard. Succinate dehydrogenase activity was measured by the spectrophotometric method of Arrigoni (1962), and malate dehydrogenase activity by the method of Thorne and Kaplan (1963). In both cases, enzymatic reactions were followed, using a Beckman model DU spectrophotometer equipped with a Gilford optical density converter and automatic sample changer. Activities were calculated from the initial zero order rates.

Electron Microscopy

For electron microscopy, mycelia were fixed by a modification of the procedure of Kellenberger, Ryter, and Séchaud (1958). Sodium chloride was omitted from the buffer mixture, and the prefixation step with 0.1 per cent osmium tetroxide was eliminated. Specimens were embedded in Epon (Luft, 1961) and sectioned on a Porter-Blum microtome, using a Dupont diamond knife. After staining with lead (Karnovsky, 1961), the sections were examined in the Siemens Elmskop Ia.

RESULTS

In earlier experiments (Luck, 1963 *a*, 1963 *b*), we found that the *chol-1* strain grows at maximal rate in shaken liquid cultures supplemented with choline chloride in concentrations varying from 1 to 100 μg/ml. The experiment shown in Fig. 1 indicates that, despite the uniformity of growth over this concentration range, incorporation of radioactive choline into a cellular lipid extract does not reach a maximum until the choline concentration in the medium is 10 μg/ml. The data indicate that when the concentration of choline is increased from 1 μg/ml (the level required for maximal growth) to 10 μg/ml (the concentration required for maximal incorporation), there is a tenfold increase in the relative amount of radioactive choline incorporated into cellular lipid extracts. In view of these findings, we chose to study the mitochondria isolated from cultures supplemented with 1 and 10 μg/ml choline chloride.

A striking difference appeared when mitochondria obtained from these two different cultures were isolated by centrifugation in a continuous sucrose density gradient. As shown in Fig. 2, mitochondria obtained from cultures at 1 μg/ml choline formed a band at an apparent density significantly higher than that of those obtained from 10 μg/ml cultures. The position of the bands was not altered when centrifugation was prolonged from 1.5 to 8 hours, and their identity as mitochondria was established by electron microscopy and by studying the distribution of cytochrome oxidase throughout the gradients. The peak of cytochrome oxidase activity coincided with the center of the visible band, and occurred at a sucrose concentration equivalent to a density of 1.22 in the case of the 1 μg/ml choline mitochondria; the corresponding value was 1.18 for the 10 μg/ml choline mitochondria.[2] As Fig. 2 further indicates, artificial mixtures of the two populations were resolved by gradient centrifugation.

Table I gives the phospholipid content of the two types of mitochondria, and indicates that the phospholipid-phosphorus to protein ratio of 10 μg/ml mitochondria is twice the value obtained with 1 μg/ml mitochondria. These data are compatible with the density differences observed, but at present cannot be used to account entirely for them. The equilibrium position taken by mitochondria in density gradients is not only influenced by the density of their components, but by interaction of the particles with the water and sucrose of the medium (de Duve, 1959). Beaufay and Berthet (1963) concluded, from studies of liver mitochondria, that these structures contained two compartments, one permeable and the other impermeable to sucrose. Changes in the apparent density of a mitochondrial band might reflect changes in sucrose permeability as well as changes in the density of mitochondrial components.

Data on the radioactivity of the lipid extracts (Table I) suggest that the difference in choline content (9-fold) of the two types of mitochondria is far greater than the difference in phospholipid content.

Some enzymatic studies were carried out to characterize the protein component of the two mitochondrial preparations. The results (Table I)

[2] The position of the band of 10 μg/ml choline mitochondria defines the lower density limit. In an experiment similar to the one outlined in Fig. 1, in which cultures were supplemented with 2, 6, 8, 10, and 20 μg choline chloride/ml, minimum density of the mitochondrial band, and maximum incorporation of radioactive choline into mitochondrial lipids was observed at 8 μg/ml, without change at higher evels.

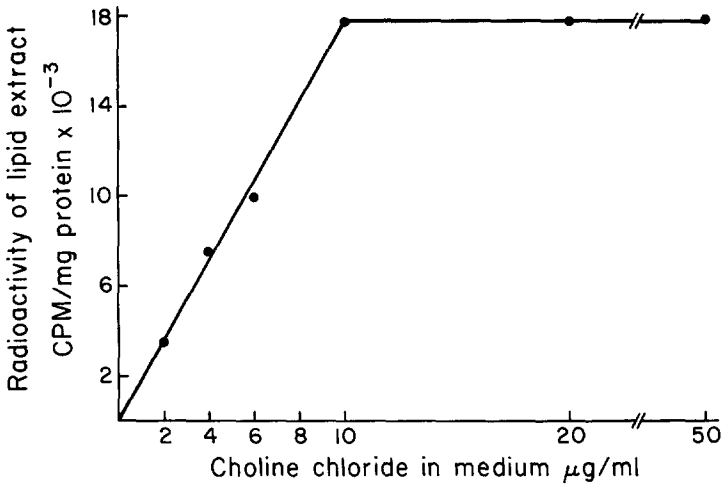


FIGURE 1 Incorporation of ^{14}C methyl-choline into cellular phospholipids. 7×10^5/ml conidia of strain 34486 were inoculated into flasks of minimal medium, supplemented with radioactive choline chloride at the various concentrations shown on the abscissa (specific radioactivity of choline added to all flasks was the same). At the end of 15 hours' culture, mycelia were collected by filtration, ground with sand in 0.44 M sucrose (0.001 M EDTA), and nuclei and cell debris were sedimented in a low speed centrifugation. Aliquots of the nuclear supernate were analyzed for total protein and for radioactivity present in the lipid extract.

TABLE I

Functional Characteristics of Mitochondria

	Mitochondrial properties					
			ΔE/5′/mg Protein			Q_{O_2} of mycelia
Cultural conditons choline chloride concentration	Phospholipid P μg/mg protein	Lipid-extractable radioactivity CPM/ mg protein $\times 10^{-3}$	Malate dehydrogenase	Succinic dehydrogenase	Cytochrome oxidase	μLO_2/mg dry mass
1 μg/ml	8.0	13.4	46.0	12.4	7.1	41.3
10 μg/ml	16.9	112.0	45.4	12.1	19.0	39.4

Data were taken from representative experiments. Mitochondria were obtained by gradient centrifugation from cultures grown 14 to 15 hours under the conditions indicated. Lipid-extractable radioactivity was determined in experiments in which methyl ^{14}C choline was the supplement. Q_{O_2}'s were determined on intact mycelia (14- to 15-hour cultures) suspended in the appropriate media.

indicate that the specific activity (relative to protein) for malate dehydrogenase and succinate dehydrogenase were identical. Only in the case of cytochrome oxidase did the 10 μg/ml choline mitochondria show a specific activity about twice that obtained with 1 μg/ml choline preparation. Since it is known (Smith and Camerino, 1963) that difficulties may be encountered in assaying the cytochrome oxidase of intact mitochondria with reduced cytochrome *c* as substrate (probably because of limitations in substrate accessibility), we measured cytochrome content by examining the mitochondrial difference spectrum. These studies (Fig. 3) indicated that, on a protein basis, the content of cytochromes a–a_3, b, and c were the same in mitochondria isolated from 1 and 10 μg/ml choline cultures.

The fact that cultures grow on 1 and 10 μg/ml choline at maximal logarithmic growth rates, and the observation that the Q_{O_2}'s of these cultures are the same (Table I) suggest that the two types of mitochondria are functionally equivalent. The data on malate dehydrogenase, succinate dehydrogenase, and cytochrome content support

this suggestion. It is not clear whether deficiency of cytochrome oxidase *activity* in mitochondria at low choline levels is an artifact of the assay procedure, or a true functional deficiency resulting from decreased content of phospholipids. The importance of phospholipids as a cofactor in electron transport as well as dehydrogenase activities is well documented in the literature (Sekuzu, 1961; Das, 1962; Fleischer, 1962).

These observations on mitochondria obtained from cultures grown at two different concentrations of choline reveal that the available amount of a lecithin precursor can strikingly alter the apparent density, the phospholipid to protein ratio, and the choline content of mitochondria. The protein components of these mitochondria, to the extent that they can be characterized by the specific activity of two dehydrogenases and content of cytochromes (determined by difference spectra), appear to be the same. These findings, taken together with the conclusion that mitochondria grow by accretion (Luck, 1963 *a*, *b*), suggest that during the growth process, incorporation[3] of protein and phospholipid into mitochondria are separate processes which can vary independently.

Mitochondrial Structure

Electron microscopic studies of cultures grown with supplements of 1 and 10 μg/ml choline chloride indicate that there are significant differences in the structure of their mitochondria. A comparison of Figs. 4 and 6 *A* (1 μg/ml choline) with Figs. 5 and 6 *B* (10 μg/ml choline) indicates that, at low choline, mitochondrial profiles appear larger and less numerous. Another characteristic of low choline cells is the presence in both the nucleus and the cytoplasm of inclusions having the appearance of lipid. The appearance of the mitochondria can be correlated with the difference in phospholipid to protein ratio. In the case of 1 μg/ml mitochondria, the ratio of surface membrane to volume is smaller than that for 10 μg/ml mitochondria.

The difference in size of mitochondria of low and high choline cultures can also be seen in electron micrographs of sections through the isolated mitochondrial fractions. Electron microscopy of low choline cultures 15, 30, and 45 minutes after addition of choline (10 μg/ml) to the medium, clearly shows a gradual transformation in mitochondrial form. At 45 minutes, the mitochondria resemble those typical of high choline (10 μg/ml) cultures.

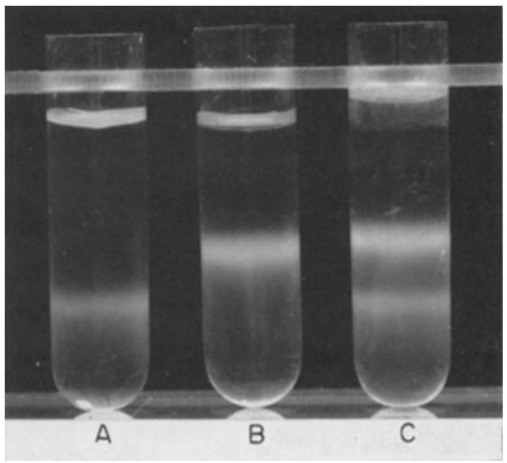


FIGURE 2 Sedimentation in sucrose gradients of mitochondria derived from cultures at 1 and 10 μg/ml choline chloride, shown in a photograph of the centrifuge tubes. Mitochondria derived from 15-hour cultures in 1 (tube *A*) and 10 (tube *B*) μg/ml choline chloride were prepared as a crude fraction by differential centrifugation, resuspended in 0.44 M sucrose (0.001 M EDTA), and layered over a continuous sucrose gradient (1.9 to 0.9 M sucrose, 0.001 M EDTA). The figure shows the appearance of the tubes after 1.5 hours' centrifugation at 39,000 RPM in a Spinco 39 SW rotor. Tube *C* was layered with an equal mixture of both types of mitochondria. The position of the bands was not changed in experiments in which centrifugation time was prolonged to 8 hours.

Changes in Mitochondria Following Addition of Choline to Low Choline Cultures

If incorporation of protein and phospholipid into the growing mitochondrial mass are separate processes capable of independent variation, it

[3] In interpreting these and subsequent experiments, the term incorporation is used in an operational sense, without specifying the mechanism by which macromolecules are added to mitochondria. At present, there is insufficient evidence to permit a choice between the possibility that proteins and lipids are synthesized from small precursor molecules *in situ*, and the possibility that they are synthesized as macromolecules elsewhere in the cytoplasm and are transported to the mitochondrion for final assembly into mitochondrial structure.

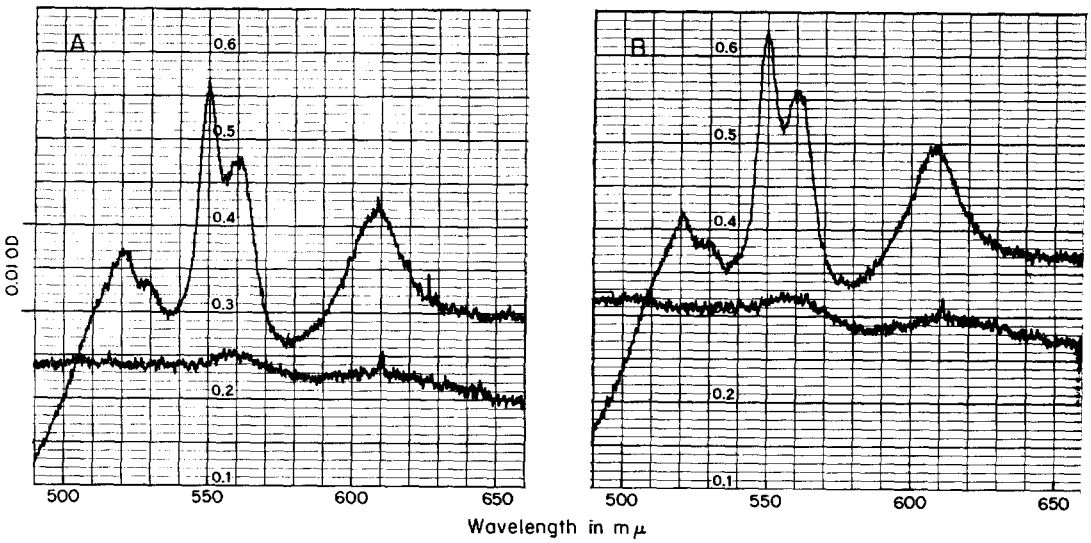


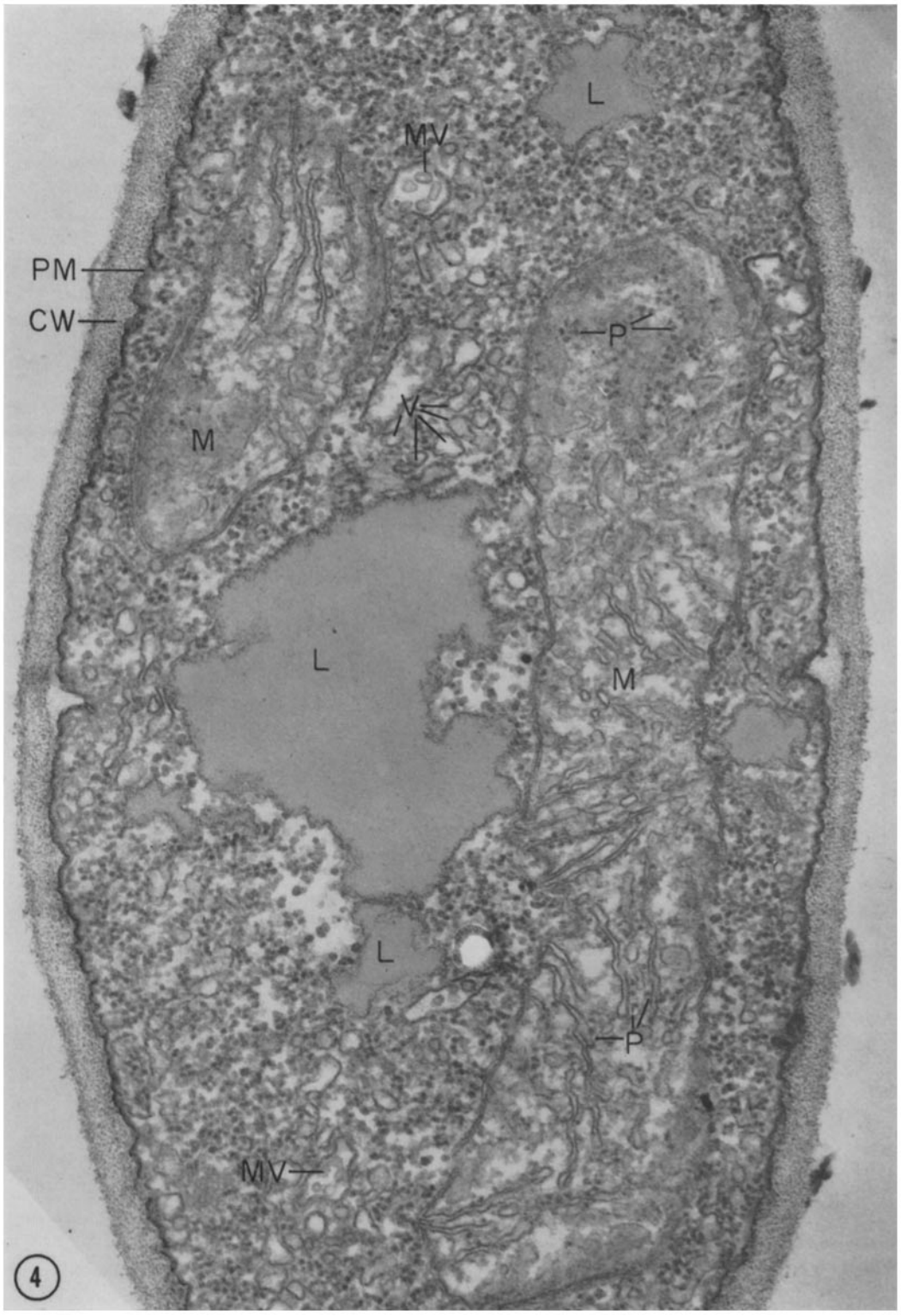
FIGURE 3 Mitochondrial difference spectra, dithionite reduction. Mitochondria were collected as bands after centrifugation in a sucrose gradient and supplemented with phosphate buffer (pH 7.4) to a final concentration of 0.033 M. The suspensions were sonicated for 30 seconds with a Branson Sonifier at a setting of 8, and Na deoxycholate (pH 7.8) was added to a final concentration of 4.5 mg/ml. Spectra were obtained with a Cary model 14 MR recording spectrophotometer equipped with a 0.1 to 0.2 slidewire. In each case, the tracing obtained with addition of dithionite to one cuvette has been superimposed on the base line. *A*, Mitochondria obtained from a 15-hour culture at 1 μg/ml choline, protein concentration 2.05 mg/ml. *B*, Mitochondria obtained from 15-hour culture at 10 μg/ml, protein concentration 2.25 mg/ml. The tracings show an α peak for cytochrome *a* to a^3 at 609 mμ, and α peaks for cytochromes *b* and *c* at 560 and 550 mμ, respectively. In addition, there are β peaks at 528 mμ for cytochrome *b*, and at 520 mμ for cytochrome *c*.

should be possible to observe this independence when more choline is added to a culture growing logarithmically at a low choline concentration.

Fig. 7 shows the result of such experiments carried out to determine rates of incorporation of choline into mitochondrial lipids when cultures growing at 1 μg/ml choline were supplemented with radioactive choline at a concentration (10 μg/ml) adequate for maximal incorporation. The experiments indicate that the doubling time for choline incorporation is ~ 50 minutes. The data apparently represent true incorporation of choline into lipids rather than transfer of methyl groups, since the results obtained with methyl-labeled choline and chain-labeled choline are in good agreement.

To obtain evidence concerning the rate of increase of mitochondrial protein during conversion

FIGURE 4 Electron micrograph showing an oblique section through a hyphal filament of the *chol-1* mutant of *Neurospora* after 15 hours' growth in minimal medium supplemented with choline chloride, 1 μg/ml. The section shows two mitochondrial profiles (*M*) against a background of small, uniform, dense ribosomal particles. Within the mitochondrion there are small particles (*P*) which bear some resemblance to cytoplasmic ribosomes. Irregularly shaped inclusions (*L*), which have the appearance of lipid, are present in the cytoplasm. These inclusions, often of large dimensions, are a typical feature of low choline cells, and are found in the nucleus as well as in the cytoplasm. The cell is surrounded by a plasma membrane (*PM*) and a cell wall (*CW*). The cytoplasm contains vesicular elements (*V*) and multivesicular bodies (*MV*). The appearance of the mitochondria is typical of low choline cells, and should be compared with those of Fig. 5. × 58,000.


L
MV
PM
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4

from low to high choline, we carried out a washout experiment using radioactive choline as a mitochondrial label, and measuring the decrease of the ratio, lipid radioactivity to protein. If choline serves as a stable mitochondrial label,[4] the rate of decrease of this ratio could be used to calculate the doubling time of protein incorporation into mitochondria. The results of this experiment, shown in Fig. 8, suggest that this value is ~ 150 minutes. Fig. 8 also indicates that ~ 150 minutes is the dry mass doubling time of whole cultures converted from low to high choline.

These results indicate that when the level of choline available to the culture is abruptly increased, this lecithin precursor is incorporated into mitochondria at a rate exceeding the rate of protein incorporation. Protein incorporation appears to go on at the same rate as the dry mass growth of the culture, and does not seem to be influenced by the rapid incorporation of phospholipids. As would be expected, this disparity in rates of incorporation results in prompt alteration of the phospholipid to protein ratio (see Table II).

Experiments with a Leucine-Requiring Strain

Our studies with the choline-requiring strain indicated that the availability of this lecithin precursor could alter the ratio of phospholipids to protein in mitochondria. To test the possibility that an analogous situation might occur in a mutant strain requiring a protein precursor, we have carried out preliminary experiments with a leucine-deficient culture. In anticipation of future experiments, we have made these studies in a doubly deficient, leucineless (D 221), cholineless (34486) strain. As in the case of the choline studies, we studied the effect of leucine supplements on the density of mitochondria, starting from the lowest concentrations which supported maximum growth rates. The results of such experiments, illustrated in Fig. 9, show that there are detectable increases in mitochondrial density when the leucine content of the medium is raised to values two and four times higher than the basal level required for maximum growth rates. These shifts in apparent density can be correlated with shifts in phospholipid to protein ratio (see legend of Fig. 9).

These findings suggest that available levels of protein precursors as well as phospholipid pre-

TABLE II

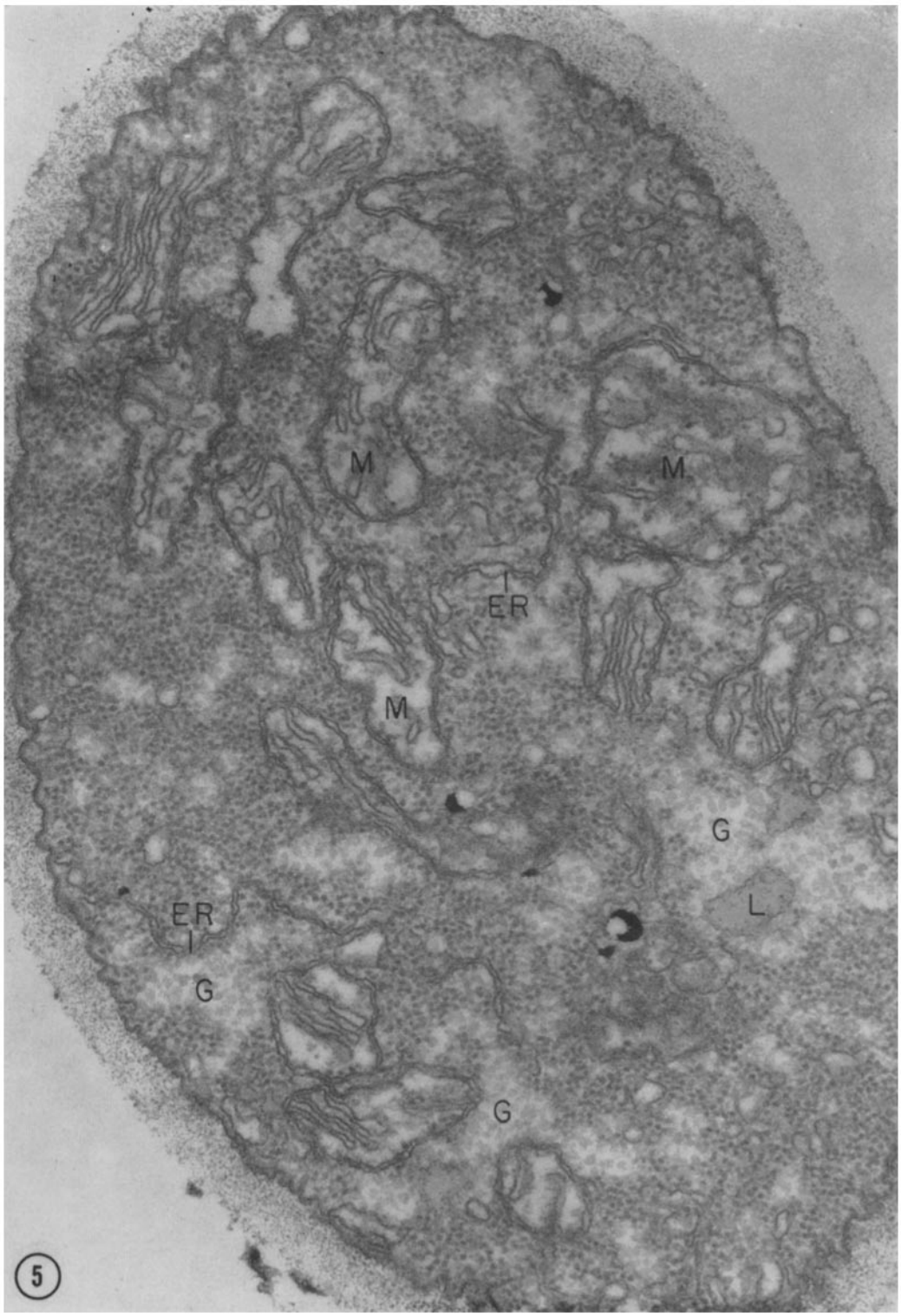
Change in Mitochondrial Phospholipid to Protein Ratio in Cultures Converted from Low (1 μg/ml) to High (10 μg/ml) Choline

Time after addition of supplemental choline	μg phospholipid-phosphorus/mg protein	
	Exp. 1	Exp. 2
0	8.1	8.0
20′	8.6	8.2
40′	10.0	9.6
60′	11.5	11.1
90′	12.0	11.6

Experimental procedure identical to that of Fig. 6. Data are given for two separate experiments.

[4] Evidence that in this type of experiment choline can serve as a stable mitochondrial label was presented in an earlier publication (Luck, 1963 *b*). To this evidence we can add the following observation: A choline-leucine–requiring strain of *Neurospora* was grown on complete medium containing radioactive choline. After 15 hours of growth, cultures are filtered and transferred to leucine-free medium containing a large excess of unlabeled choline. During the following 110 minutes, there is no change in the mitochondrial ratio, lipid-extractable radioactivity to protein. Parallel experiments, without radioactive choline, in which radioactive lysine is present during the leucine free period, show less than 6 per cent of maximum lysine uptake (determined experimentally) during these 110 minutes.

FIGURE 5 Electron micrograph showing an oblique section through a hyphal filament of the *chol-1* mutant of *Neurospora* after 15 hours' growth in minimal medium supplemented with choline chloride, 10 μg/ml. The section shows many mitochondrial profiles (*M*) which, when compared with with Fig. 4, appear to be much smaller and more numerous. Lipid deposits (*L*) are smaller and less numerous than those seen in low choline cells. The section shows elements of agranular endoplasmic reticulum (*ER*) and polysaccharide deposits (*G*). × 58,000.


M
M
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5

cursors can influence the chemical composition of mitochondria. In both cases, the observations were made with strains of *Neurospora* which had absolute requirements for the precursors in question. It was important to determine whether a wild type strain, which required no complex chemical supplements, would respond in a similar way to additions of precursor compounds to the growth medium.

Experiments on Wild Type

Fig. 10 illustrates the results of an experiment in which large amounts of choline and large amounts of casein enzymatic hydrolysate were added to the minimal culture medium of a wild type strain (5256). It will be seen that, in terms of density and phospholipid to protein ratio, each precursor supplement has influenced the chemical composition of mitochondria in the expected direction. It follows that, even in the absence of a strict biochemical requirement, available concentrations of precursors influence the composition of mitochondria.

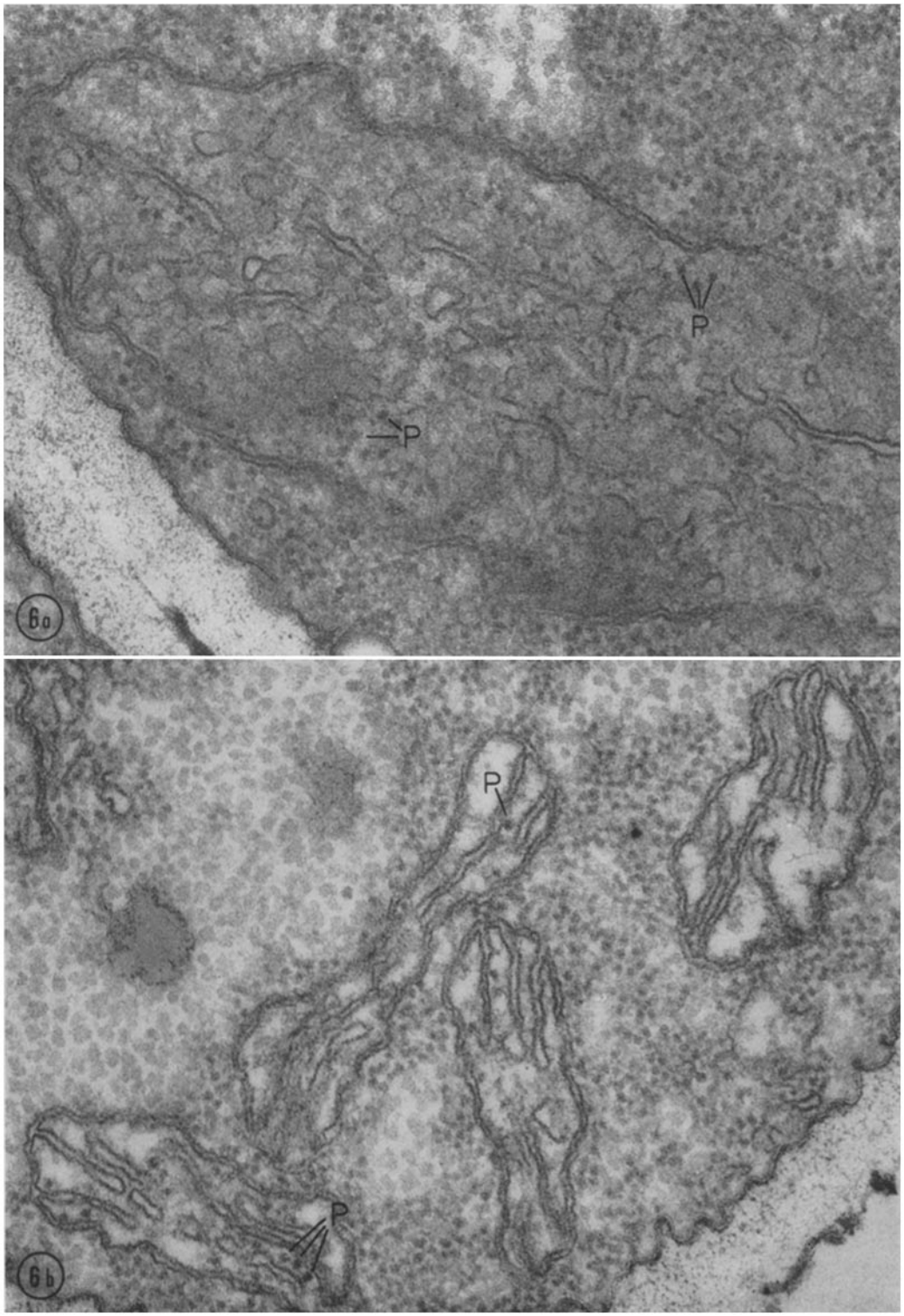
In the case of mutant strains with specific biochemical requirements, available pool size of a required precursor is largely determined by the level of supplementation to the culture medium. The situation is distinctly different in wild type strains growing on a single carbon source (sucrose), for here, pool size is influenced by the complex of reactions which produce and utilize the various precursors. The fact that adding choline or amino acids to the medium of a wild type strain influences the mitochondrial phospholipid to protein ratio suggests that, even here, rates of incorporation can be influenced by the available level of precursors. We checked this point by determining the time course of choline incorporation into mitochondria when wild type cultures (5256) growing in minimal media were supplemented with radioactive choline chloride (20 μg/ml). In this experiment, similar in design to that of Fig. 7, the doubling time for choline incorporation was $\sim$ 90 minutes. In an experiment similar to that of Fig. 8, culturing wild type with radioactive choline chloride 1 μg/ml and diluting label by adding 100 μg/ml unlabeled choline, we obtained an estimate of the doubling time of protein incorporation ($\sim$ 170 minutes). These results give additional support to the conclusion that, in wild type also, rates of incorporation of phospholipid and protein can vary independently in response to variations in the concentration of the respective precursors.

DISCUSSION

The results show clearly that the composition of mitochondria in exponentially growing *Neurospora* can be varied within relatively wide limits. The variation affects the ratio of phospholipid to protein, and the density as determined by isopycnic centrifugation. It can be brought about by changes in the incorporation of either phospholipids or protein into mitochondria. That is, a decrease in the ratio, phospholipid to protein, can be the result of conditions which limit phospholipid synthesis (limited choline supplementation to a choline-requiring mutant) or those which favor protein synthesis (increasing leucine supplementation to a leucine-requiring mutant).

When these findings are considered together with the evidence (Luck, 1963 *a*, *b*) that the mitochondrial mass grows by addition of new components to existing *mitochondrial* structures, it is apparent that changing composition must reflect independent variation in the rates of incorporation of protein and phospholipid into mitochondria. This independent variation was actually demonstrated in the case of a choline-requiring strain, when the level of choline supplementation was abruptly increased. The change in choline availability resulted in an immediate and rapid incorporation of choline into the mitochondrial lipids; while protein incorporation went on at a rate equal to the growth rate of the culture. The disparity in the rates of these processes was associ-

FIGURE 6 Electron micrographs at high magnification of mitochondria from cultures at two levels of choline supplementation. *A*, 1 μg/ml choline chloride. *B*, 10 μg/ml choline chloride. At higher magnification the differences, already described, between mitochondria of cultures at low and high choline supplementation can be clearly seen. The average diameter of low choline mitochondria is 0.6 to 0.7 μ, while for high choline mitochondria the value is 0.2 to 0.25 μ. Intramitochondrial particles (*P*) are visible. × 90,000.


P
P
6a
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P
6b

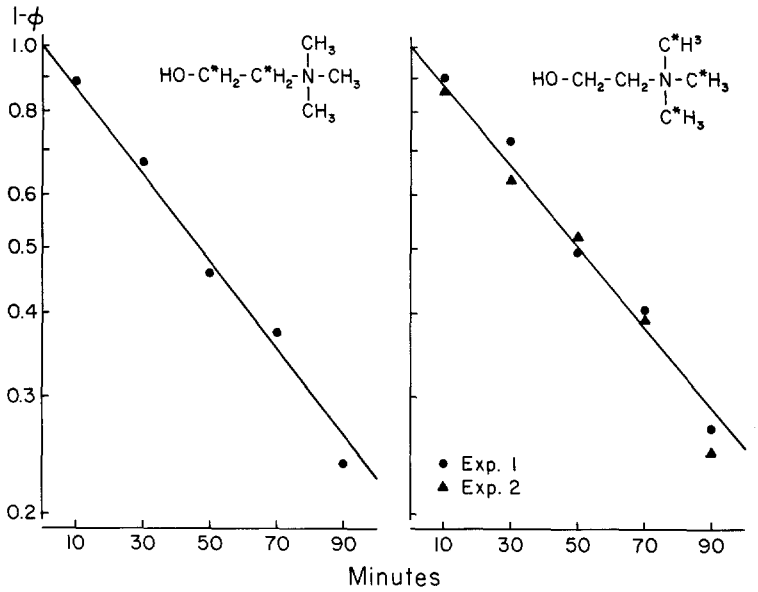

FIGURE 7 Time course of incorporation into mitochondrial lipids of chain-labeled and methyl-labeled ^{14}C choline, during shift from low choline to high choline medium. Conidia were cultured in flasks containing 100 ml of 1 μg/ml choline media, according to the usual procedure. After 15 hours of growth, radioactive choline to a final concentration of 10 μg/ml was added to the medium. At the time points indicated, culture flasks were chilled, and their mycelia collected by filtration in Buchner funnels. Mitochondria were isolated by gradient centrifugation, and their lipid-extractable radioactivity and total protein were determined. Results are plotted, according to the suggestion of Britten (1962), as $1 - \phi$ against time, in which ϕ is the ratio of specific activity (lipid-extractable counts per mg protein) for any time to the specific activity obtained on prolonged growth in label (medium concentration, 10 μg/ml radioactive choline chloride).

ated with an immediate and continuing increase in the mitochondrial phospholipid to protein ratio.

It is significant that chemical composition and density of mitochondria obtained from wild type cultures can also be altered simply by adding choline or amino acids to the sucrose medium. In a wild type culture growing in minimal sucrose medium, these cell structures are produced from a single carbon source, and the available level of any precursor is determined by the many complex reactions which produce, and draw on, intracellular pools. In this respect, the situation is somewhat different from that encountered in biochemical mutant strains in which availability of a required precursor is largely determined by the extent of supplementation to the medium. Yet the effect on mitochondrial composition of adding supplements is comparable in both mutant and wild type strains. This suggests that cellular regulation of mitochondrial composition operates, in part, at the level of synthesis of the major components, protein and phospholipids. The fact that it is possible to influence incorporation processes and the mitochondrial composition by providing precursor supplements to wild type strains, indicates that these synthetic processes normally operate at substrate levels below those required for maximal velocity.

It can be assumed that the upper limit for influencing mitochondrial composition by stepping up the synthetic rates would be that concentration of precursor in the medium which saturates the synthetic mechanism in the cell. In the case of a choline-requiring strain, we did observe that addition of choline to the medium in concentrations higher than 8 μg/ml (for 15-hour cultures) produced no further change in the density of mitochondria nor in the lipid choline to protein ratio. It is not clear, however, whether in this situation the limiting mechanism is saturation of a lecithin synthetic pathway, or an independent restriction

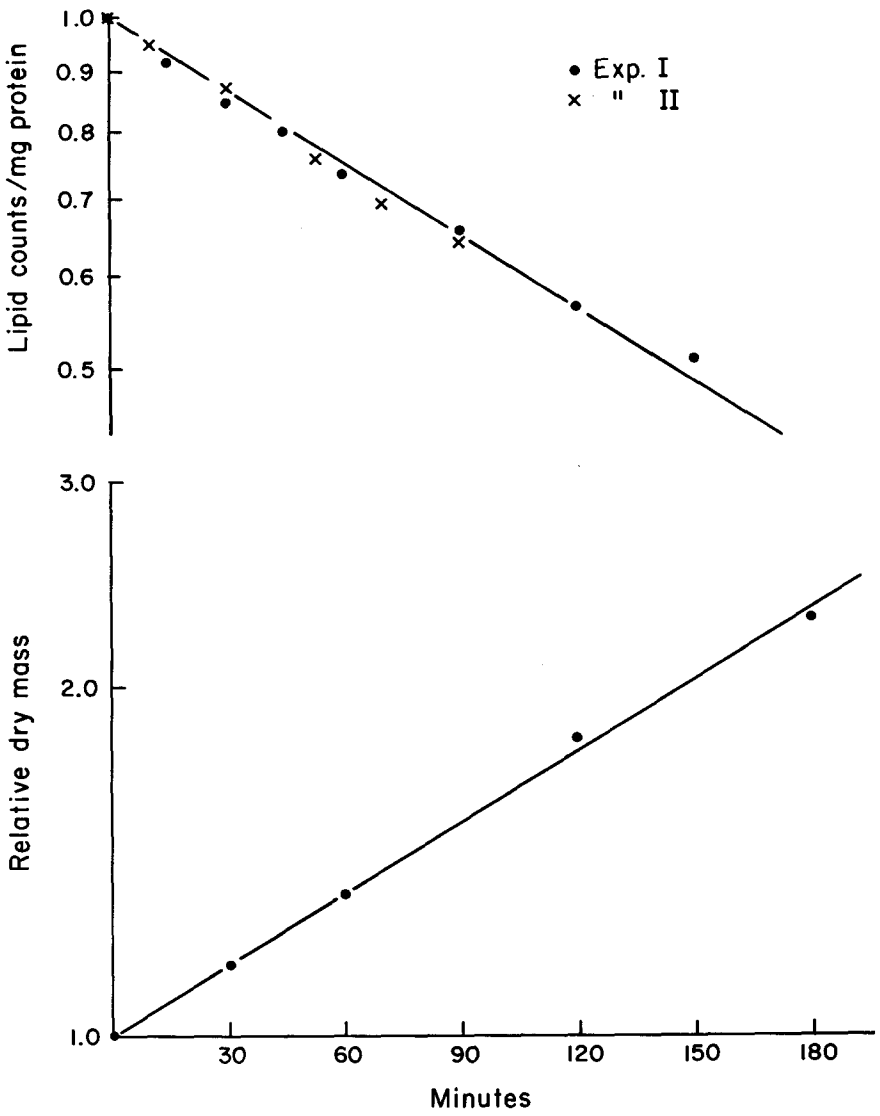

FIGURE 8 Decrease in mitochondrial "specific radioactivity" during a washout experiment.

Cultures were grown in 100 ml of medium containing methyl ^{14}C choline chloride, 1 μg/ml, according to the usual procedure. After 15 hours of growth, non-radioactive choline (100 μg/ml final concentration) was added to the flasks. At various time points, mitochondria were obtained from entire cultures and the ratio, lipid-extractable radioactivity to protein, was determined. Since choline serves as a stable mitochondrial label (see footnote 4), the time course of dilution indicates the rate of increase of the mitochondrial protein mass.

The lower curve, taken from a parallel experiment, gives the relative increase in dry mass (the value at zero time taken as 1.0) of whole cultures during the change in medium concentration of choline chloride from 1 to 100 μg/ml.

imposed by the number of sites available for phospholipid in the organization of mitochondria.

Results of experiments now in progress seem to have some bearing on this question. A doubly deficient, choline- and leucine-requiring strain was grown in a medium with choline and leucine supplements for 15 hours. At that time, the cultures were filtered and transferred to choline-enriched,

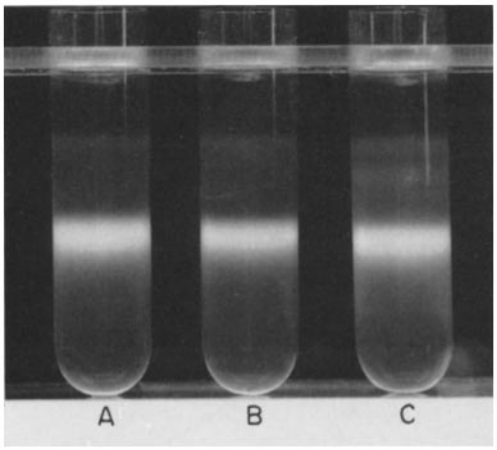

FIGURE 9 Sedimentation in sucrose gradients of mitochondria derived from a leucine-requiring strain shown in a photograph of the centrifuge tubes. Cultures of a *chol-1, leu-1* strain were grown in minimal medium with the following additions of DL-leucine: *A*, 0.25 mg/ml (this is the lowest concentration which supports growth at maximal rates); *B*, 0.50 mg/ml; *C*, 1.0 mg/ml. The choline chloride concentration was 10 μg/ml in all cases. After 15 hours of growth, mitochondria were isolated as crude fractions from each culture, and isopycnic centrifugation was carried out according to the procedures of Fig. 2. In two separate experiments, the phospholipid to protein ratios of the mitochondrial bands were:

Culture conditions	μg phospholipid-phosphorus/mg protein		
	A	*B*	*C*
Exp. 1	16.2	15.3	13.2
Exp. 2	17.5	15.4	12.8

leucine-free medium. The results showed that radioactive choline incorporation could go on for 110 minutes in the absence of significant protein incorporation. During this period, the density of mitochondria decreased and the phospholipid to protein ratio reached a maximum value of 19 μg phospholipid-phosphorus/mg protein. During a subsequent period (110 to 150 minutes), there was no further incorporation of choline, but there was evidence of beginning protein incorporation. These experiments indicate that the mitochondrion can accommodate more phospholipid than that present when a cholineless strain is grown on saturating choline supplementation (phospholipid to protein ratio, 16 μg phospholipid-phosphorus/mg protein); and, accordingly, they suggest that cellular regulatory mechanisms other than those controlling the rate of synthesis are involved in the control of mitochondrial composition (phospholipid content). It must be emphasized, however, that these last experiments deal with stationary cultures and, as such, are essentially different from those which have been presented in this paper. It may be that the adjustment process seen here is not directly related to regulatory mechanisms operating in exponentially growing cells.

In the case of low and high phospholipid mitochondria produced by variations in the level of supplementation to a choline-requiring mutant, electron microscopy of intact hyphae has demonstrated a striking and characteristic difference in mitochondrial morphology. The difference appears to influence mainly the size of individual mitochondria rather than total volume of the mitochondrial population. When low choline cultures are provided with additional choline, there is a progressive change in mitochondrial size, which appears to reach the dimensions of typical high choline mitochondria in 45 minutes (less than one-third of a doubling cycle). In view of these findings, it is reasonable to suggest that, in these circumstances, phospholipid incorporation into mitochondria may have a special influence on the mitochondrial division process, permitting them to divide at a rate faster than the growth rate of the mitochondrial mass.

The changes in mitochondrial morphology seen in low choline cultures bear some resemblance to those encountered by Wilson and Leduc (1963) in their study of hepatic parenchymal cells of fatty acid–deficient mice.

At the present time, we have no evidence to suggest that variations in gross chemical composition of mitochondria have seriously altered their function. In all cases, the mitochondria supported maximal exponential growth rates of an obligate aerobic organism. In the case of the *chol-1* mutant, in which we encountered the greatest variation in mitochondrial composition (a twofold difference in phospholipid to protein ratio and a ninefold difference in lipid choline to protein), the Q_{O_2}'s of the two types of culture were similar. The protein-specific activity of malate and succinate dehydrogenases and the content of cytochromes were the same. Only in the case of cytochrome oxidase

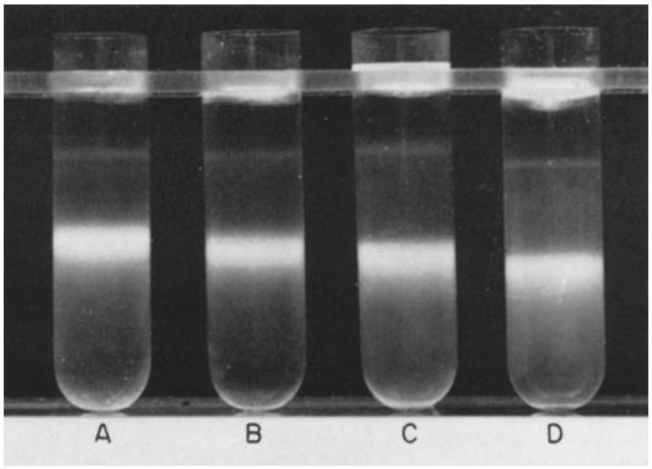

FIGURE 10 Sedimentation in sucrose gradients of mitochondria derived from a wild type strain, shown in a photograph of the centrifuge tubes. Cultures of a wild type strain were grown in minimal media with the following additions: *A*, choline chloride, 100 μg/ml; *B*, no additions; *C*, casein hydrolysate (enzymatic), 1 mg/ml; *D*, casein hydrolysate, 1.5 mg/ml. After 15 hours of growth, mitochondria were isolated as crude fractions from each culture, and isopycnic centrifugation was carried out according to the procedures of Fig. 2. In two separate experiments, the phospholipid to protein ratios of the mitochondrial bands were:

	μg phospholipid-phosphorus/mg protein			
Culture conditions	*A*	*B*	*C*	*D*
Exp. 1	16.4	13.0	11.7	10.5
Exp. 2	16.5	13.1	11.5	10.5

activity, in which assay procedures could be questioned, was there a detectable difference in the two types of mitochondria. These findings support the tentative conclusion that the differences we have seen are an indication of permissible variation in the gross chemical (phospholipid) composition of functioning mitochondria. Such permissible variation in lipid content is already suggested by the observations of Fleischer *et al.* (1962), who studied restoration of electron-transport activity in mitochondria depleted of phospholipid by solvent extraction. Their results showed that electron-transport activity could be completely recovered when added phospholipid was bound to mitochondria in amounts lower than those originally present; in addition, there appeared to be no requirement for specific phospholipids (cardiolipin, phosphatidylcholine, phosphatidylethanolamine, or mixed mitochondrial lipids) to restore respiratory activity.

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