Cytochrome Synthesis in Synchronous Cultures of the Yeast, Saccharomyces cerevisiae*

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SUMMARY

The synthesis of cytochromes aa₃, b, and c has been investigated during synchronous growth in the yeast, Saccharomyces cerevisiae. These cytochromes increase in concentration continuously throughout each cell cycle, with an approximate doubling in rate during successive cycles. The rates of cytochrome formation are considerably higher in galactose-grown cultures than in cells grown in glucose. Although cytochrome aa₃ increases at a continuous rate, its functional counterpart, cytochrome c oxidase, increases in a stepwise fashion, with the increments occurring at the beginning of each new cell cycle. Chloramphenicol, a specific inhibitor of intramitochondrial protein synthesis, inhibits the formation of cytochrome aa₃ at all stages of the cell cycle, but does not inhibit cytochrome c. Chloramphenicol exhibits a somewhat intermediate effect on cytochrome b synthesis, with transient inhibition occurring only when the drug is added prior to or during the initial part of the first cell cycle. After this time, chloramphenicol had no effect on the rate of cytochrome b synthesis. The data indicate that under our conditions of cell synchrony mitochondrial membrane formation as reflected by increments in mitochondrial cytochromes occurs by continuous accretion of new material throughout the cell cycle. Intramitochondrially synthesized polypeptide products, responsible for the formation of new cytochrome aa₃, appear to be synthesized throughout the cell cycle.

The study of synchronously growing cells has proved to be an exceptionally useful means for the examination of events occurring at different times during the cell cycle (1). Although a wide variety of cellular phenomena have been studied in synchronous cells, relatively few investigators have analyzed the behavior of enzymes specific to a particular organelle (2–6). Since growth during the cell cycle appears to consist of a series of highly ordered and reproducible chemical events, the study of specific organelle markers during repetitive synchronous cell cycles should help elucidate patterns of organelle assembly. This experimental system appears particularly well suited for the study of various aspects of mitochondrial biogenesis.

It has been established that the respiratory chain components, cytochromes aa₃, b, and c, are structural elements of the inner mitochondrial membrane (7–9). A large body of evidence indicates that some of the polypeptides necessary for the normal structure and function of two of these cytochromes (aa₃ and b) are the products of an intramitochondrial protein synthesizing system thought to be directed by the unique genetic information content of the mitochondrion (for reviews see Refs. 10–12). The present investigation was undertaken to examine the behavior of cytochromes aa₃, b, and c and the functional counterpart of the cytochrome aa₃ complex, cytochrome c oxidase, during successive cell cycles in synchronously growing cultures of the yeast, Saccharomyces cerevisiae. The timing of intramitochondrial polypeptide synthesis was also investigated by an examination of the effects of the addition of chloramphenicol at various times during the cell cycles on cytochrome synthesis. Based upon its ability to inhibit amino acid incorporation into protein by isolated mitochondria, chloramphenicol is considered to be a selective inhibitor of polypeptide synthesis taking place on mitochondrial ribosomes (13–15).

EXPERIMENTAL PROCEDURE

Conditions for Cell Synchrony and Culture—The yeast used in this work, a diploid strain of Saccharomyces cerevisiae designated iso N, was obtained from Dr. C. J. Avers (16). Cells were synchronized by the method of Williamson and Scopes (17) with several modifications (3). Synchronous cultures were grown on the medium of Scopes and Williamson (18), which was modified to contain 0.2% yeast extract with either 2% glucose or 2% galactose as the carbon source. Large scale synchronous cultures of up to 24 liters were grown at 25.0° with vigorous agitation in tandemly arranged F-14 fermentor jars in an FS-314 fermentor drive assembly (New Brunswick Scientific Co.). Cultures were inoculated to an initial cell density of 2 g wet weight cells per liter for standard cytochrome quantitation experiments, and either 0.5 g or 2 g of cells per liter, wet weight, for chloramphenicol inhibition experiments. Although not entirely satisfactory from all points of view,
this method of obtaining synchronous cultures was chosen for several reasons. The method has been used in previous studies of the pattern of accumulation of respiratory components (8) and phospholipids (1) it enables one to study two or three successive cell cycles (a necessity for these investigations). It provides the relatively large yield of synchronous cells needed for the cytochrome determinations and not readily provided by other methods of obtaining synchronous cultures.

Methods for Monitoring Cell Synchrony—The quality of cell synchrony was determined by three independent methods. (a) Direct morphological observation of the cells at random intervals indicated the time of onset of budding. (b) Total cell DNA was measured in 40-ml aliquots of culture removed at 15-min intervals. Cell growth was inhibited with 1 ml of 37% formalin; the cells were harvested by centrifugation, and the DNA was extracted and measured according to the method of Burton (19). (c) Changes in cell density indicated by the ratio of dry weight to cell volume were also employed to measure the time of onset of bud formation, and the quality of cell synchrony. Packed cell volumes were measured by centrifugation into graduated conical centrifuge tubes. Dry weights of smaller samples were determined by desiccation of the washed cell samples overnight at 80° in an oven.

Quantitation of Cytochromes—One-liter samples were removed from glucose- or galactose-grown cultures at 15- or 20-min intervals, washed, and resuspended in chilled distilled H2O. Cell samples were stored on ice until the completion of each experiment. Individual cell samples were then resuspended in chilled distilled H2O to a final cell density of 0.3 g of cells per ml, wet weight. Approximately 5 ml aliquots of each sample were reduced with dithionite and placed in a 0.5-cm light path cuvette designed after that described by Claasie et al. (20), and immediately immersed in liquid nitrogen. After returning liquid nitrogen temperature (77 K), the cuvette was placed in a Dewar flask-cuvette holder assembly also designed after that of Claasie et al. (20). The frozen cell suspension was scanned from 630 to 540 nm in a Cary model 14 spectrophotometer in the 0 to 1.0 absorbance sensitivity range. The sample cuvette was blanked against a series of Zeiss neutral filters. Absorbance differences between absorption maxima and isobestic points for cytochromes a, b, and c were determined (cytochrome a, 599 to 630 nm; cytochrome b, 558.5 to 575 nm; cytochrome c, 547 to 540 nm) (Fig. 1). Essentially, no differences were observed in repeated scans of each aliquot of the same cell sample, and approximately ±5% deviations in absorbance (maxima-isobestic points) were obtained in scans of different aliquots of the same cell sample. All of the cytochrome absorbances represent values calculated from the mean of three scans, each obtained from a different aliquot of the same cell sample. Relative cytochrome concentrations were computed employing the following room temperature molar extinction coefficients: cytochrome aα, 24.0 mm−1 cm−1 (21); cytochrome b, 20.0 mm−1 cm−1 (22); cytochrome c, 19.0 mm−1 cm−1 (23). To correct these values for light scattering effects and liquid nitrogen temperature augmentation of the absorption bands, the following procedure was employed. For the construction of cell samples containing varying concentrations of cytochromes, different proportions of exponential phase cells grown on 10% glucose (highly repressed low cytochrome content) were mixed with stationary phase cells grown on 2% ethanol (derepressed cells with high cytochrome content). A small aliquot of each of the cell mixtures was scanned at liquid nitrogen temperature (77 K), and cytochrome c was quantitatively extracted from the remainder of each cell mixture according to the procedure of Sels et al. (24). From plots of the "extracted" cytochrome c concentrations against the values determined by low temperature spectroscopy of the whole cells (Fig. 2A), a series of correction factors were calculated (Fig. 2B). Scans for cytochrome concentration were always made at the same cell concentration used to construct the standard correction curves. In experiments where cytochrome concentrations were measured during synchronous growth, we have assumed that the factor correcting cytochrome c is valid for the other cytochromes as well. Thus, the 77 K whole cell values for cytochromes aα and b were corrected by the factors determined for cytochrome c. However, in chloramphenicol inhibition experiments

where significant divergence between cytochrome c and the other two cytochromes was obtained, the values of aα and b were not multiplied by the cytochrome c correction factors but instead were expressed as uncorrected molar concentrations per liter of culture. Although quantitatively incorrect, these values served to indicate relative differences in cytochrome aα and b throughout cell growth.

Cytochrome c Oxidase Activity Assay At 20-min intervals, 1-liter samples were removed from the culture and immediately chilled by the addition of crushed ice. Cells were harvested by centrifugation at 0-4° and washed twice by centrifugation. The cell pellets were resuspended to 20 ml in 0.65 Tris buffer at pH 7.4, and the cell suspensions were passed twice through a chilled French pressure cell at 18,000 p.s.i. (American Instrument Company, Silver Spring, Md.). Cytochrome c oxidase activity (ferro-cytochrome c: oxygen oxidoreductase, EC 1.9.3.1) was assayed on the whole cell brei according to the method of Avers et al. (25) and expressed as the first order rate constant K−1 min−1 per liter of original culture. For expression of enzyme activity in this manner, the degree of cell breakage was monitored by direct morphological examination with phase contrast optics and by determination of the percentage of total cell protein released into the cell-free supernatant according to the method of Cottrell and Avers (4).

Chloramphenicol Inhibition—The timing of intramitochondrial polypeptide synthesis during the cell cycle was determined by pulsing individual galactose-grown synchronous cultures at different times prior to, or during the first cell cycle with 2.4 mg of chloramphenicol per ml of culture (Sigma). Under the experimental conditions employed in this study, 2.4 mg per ml was the maximum concentration of chloramphenicol that could be completely dissolved in the culture medium. That this concentration was sufficient to inhibit mitochondrial protein synthesis in this yeast strain is indicated by its effect on cytochrome aα accumulation (see "Results," Fig. 9). The iso N strain of S. cerevisiae was sensitive to chloramphenicol, as indicated by its inability to grow on ethanol in the presence of the drug. To insure that the chloramphenicol was immediately solubilized, it was first dissolved in enough methanol to make the final methanol concentration 1%. Similar amounts of methanol were added to control cultures. This method of contamination had no detectable effect on culture viability, growth, or macromolecular synthesis. Similar findings have also been obtained by Mahler and Perlman (26).

RESULTS

Cell Synchrony—The quality of synchrony in both glucose- and galactose-grown cells appeared to be excellent, as evidenced
PERCENT GLUCOSE GROWN CELLS IN MIXTURE

FIG. 2. A, relationship between "extracted" cytochrome c concentrations (△——△) and values derived from room temperature (●——●) and liquid nitrogen temperature (○——○) absorption spectrum scans of mixtures of glucose-grown and ethanol-grown cells. B, plot of correction factors necessary to convert apparent cytochrome c concentrations derived from liquid nitrogen temperature scans of whole cells to "extracted values." All values are expressed as concentration per liter of a suspension of 0.3 g of wet weight cells per ml.

FIG. 3. Total cell DNA (●——●) and cell density, i.e., ratio of dry weight to packed cell volume (○——○), during synchronous cell culture. Initiation of new cell cycles, determined by the timing of new rounds of bud formation, is designated by arrows. The data shown are from a typical glucose-grown culture. Similar data were obtained from galactose-grown cultures after an initial lag period.

by the timing of initial bud formation, increase in total DNA content, and changes in cell "density" (Fig. 3). All of the cultures were monitored through at least two consecutive cell cycles without appreciable loss in the quality of synchrony. The duration of individual cell cycles was approximately 100 min in glucose-grown cells and 140 min in galactose cultures. Variations in the duration of consecutive cell cycles on either carbon source were less than 15 min. In galactose cultures, the first round of bud formation occurred after a lag period of 5 to 6 hours. This lag has been tentatively interpreted to be the time necessary for cells synchronized in glucose to adapt to galactose. Unfortunately, it was not possible to induce synchrony in cells which had been pre-grown in galactose as the sole carbon source.

Cytochrome c Quantitation—Cytochrome c concentrations in artificial cell mixtures, determined by spectrophotometric analysis of whole cells at room temperature, were consistently 7 to 8 times higher than values obtained by means of the extraction procedure of Sels et al. (24) (Fig. 2A). These findings have been verified in three independent experiments. To insure that all cytochrome c was removed by the extraction procedure, we scanned the extracted cell residues for the presence of cytochrome c at both room and liquid nitrogen temperatures and also re-extracted them to remove any residual cytochrome c. In no instance was any additional cytochrome c detected. To test the possibility that some cytochrome c degradation took place during the extraction procedure and to check the validity of the quantitation scheme, we processed and quantitated known molar concentrations of horse heart cytochrome c (type III, Sigma) according to the procedure of Sels et al. (24). In all of the cases, calculated cytochrome c concentration values were the same as the known values. It may be concluded that there is considerable augmentation of the \(\alpha\)-absorbing band in analyses of whole cells even at room temperature.

A comparison of the absorbance of the \(\alpha\)-band maximum for cytochrome c at 77 K and at room temperature indicates a 3- to 4-fold enhancement at the lower temperature (Fig. 2A). Although it has been reported that other low temperature procedures employing aqueous glycerol have yielded 10- to 20-fold enhancements in cytochrome absorption maxima (27, 28), in our hands these procedures have yielded less consistent results than those obtained by the method described here. Fig. 2A indicates that, unlike the values obtained at room temperature, the 77 K values deviated from linearity, with intensification greater at lower cytochrome concentrations. A similar deviation from linearity was observed when the \(\alpha\) absorption maxima of different concentrations of purified cytochrome c were compared at room temperature and 77 K (29). As described under “Experimental Procedure,” a correction curve was constructed that made it possible for whole cell values of cytochrome c concentration determined at 77 K to be converted to absolute values (Fig. 2B).

The uniformity of cells in each sample and differences in cell morphology at different times in the cell cycle might lead to
differences in light scattering that could introduce errors in the measured cytochrome concentration. To test this possibility, we scanned samples obtained from different stages of a synchronous culture at liquid nitrogen temperature and quantitated cytochrome c according to the described procedure. Cytochrome c was then extracted from the remainder of each sample according to the procedure of Sels et al. (24). During synchronous growth, relative values obtained for cytochrome c concentration by the two procedures were identical at all time points examined. From these data we concluded that light scattering differences through the cell cycle did not significantly affect determinations of cytochrome c concentrations. This was assumed to hold true for cytochromes aa$_3$ and b as well.

Cytochrome Accumulation During Cell Cycle—During synchronous growth, the content of the cytochromes examined appeared to increase continuously throughout each cell cycle from an initially high base line. When cells were grown in glucose, the accumulation rate but not the content approximately doubled upon entry of the cells into each succeeding cell cycle (Fig. 4A). The accumulation rate of galactose-grown cells increased more than 2 fold on passage from the first to the second cycle (Fig. 4B). Also in the case of the galactose grown culture the content of cytochromes also doubled on passage from the first to the second cycle. Fig. 5 shows that uncorrected data, although quantitatively incorrect, also indicate a similar pattern of continuous increase in cytochrome concentration. We are not able to determine whether the increase in cytochrome content is due to a doubling of a linear rate of synthesis in each cell cycle or to a smooth exponential rate of increase. A detailed statistical analysis of this distinction by D. A. Williams in Mitchison and Creanor (30) predicts maximal differences in content of 3% for these two modes of increase. Such small differences are beyond the resolution afforded by the present experimental scheme.

Comparison of Fig. 4, A and B indicates the rate of increase in cytochromes aa$_3$, b, and c to be considerably greater in galactose- than in glucose-grown cells. These differences are even more apparent when "specific" cytochrome concentrations are calculated (concentration per g of cells, wet weight) as depicted in Fig. 6, A and B. These figures indicate that, whereas the amount of each cytochrome in the total culture increased during synchronous growth, the "specific concentration" decreased in cultures grown on glucose, which strongly represses mitochondrial
development. In cells grown on the weakly repressing galactose, specific cytochrome concentrations fluctuated a little, but did not consistently decline as did those of glucose-grown cells.

Accurate measurement of cytochrome c concentration during synchronous growth was not possible. Although the ρ absorption peak of this cytochrome was easily resolved at the beginning of each experiment (Fig. 1), later it invariably became masked by cytochromes b and c.

Analysis of the changes of cytochrome c oxidase activity during synchronous growth, indicated stepwise increase in activity (Fig. 7), with the increments occurring during the initial phase of each cell cycle. The behavior of cytochrome oxidase activity is in sharp contrast to its structural counterpart, cytochrome aa₃, which increased continuously throughout the cell cycle. Also although the amount of cytochrome aa₃ does not double in each of the two successive cell cycles shown, that of cytochrome oxidase activity does. Thus, the ratio of functional capacity per unit structure of the terminal oxidase in the electron transport chain varies during the cell cycle; a high ratio near the beginning of the cell cycle is followed by a steady decline.

Chloramphenicol Inhibition Studies—The administration of chloramphenicol at various points during the cell cycle had no detectable effects on the rate of increase of total cell DNA or wet weight. The drug had different effects on the rates of synthesis of cytochromes aa₃, b, and c. Chloramphenicol pulses (2.4 mg per ml) introduced prior to, and at approximately 20-min intervals during, the first cell cycle in individual galactose-grown cultures did not inhibit cytochrome c synthesis (Fig. 8). On the other hand, cytochrome aa₃ synthesis was completely inhibited after the addition of chloramphenicol at any point in the cell cycle (Fig. 9). Inhibition was complete within 10 min of addition of the drug. An intermediate effect of chloramphenicol on cytochrome b synthesis was noted (Fig. 10). The addition of chloramphenicol prior to or during the first half of the initial cell cycle inhibited cytochrome b accumulation only transiently, with full recovery occurring by the time of entry into the third cell cycle (Fig. 10, B and C). Of note was that the inhibitory effect of chloramphenicol on cytochrome b synthesis was delayed when added just prior to first budding (Fig. 10A) but was immediate when added during the initial part of the first cell cycle (Fig. 10C). Addition of chloramphenicol during the second half of the first cell cycle appeared to have little or no inhibitory effect on the rate of cytochrome b accumulation (Fig. 10D), and addition during the second and third cell cycles had no apparent effect.

The decreasing inhibitory effect of chloramphenicol on cytochrome b synthesis during the first cell cycle and its failure to inhibit b synthesis during the second and third cycles did not appear to be secondary to decreasing ratios of chloramphenicol concentration to cell mass during growth. When synchronous cultures inoculated with 25% of the standard-sized cell inoculum were pulsed with the same concentration of chloramphenicol used previously (2.4 mg per ml), identical patterns of cytochrome b synthesis were observed.

Further Characterization of Cytochrome b Synthesis in Presence of Chloramphenicol—Previous investigators have reported the loss of both cytochrome aa₃ and b ρ absorption peaks in glucose-grown yeast after prolonged exposure to chloramphenicol (14, 31, 22), and we have confirmed this effect in galactose-grown cells. Therefore, the continued synthesis of cytochrome b reported here was subjected to further scrutiny. To evaluate the possibility that continued cytochrome c synthesis and accumulation led to the observed increases in cytochrome b content, we added increasing amounts of horse heart cytochrome c to aliquots of a whole-cell suspension. Liquid nitrogen absorption spectrum

Fig. 6. Concentrations of cytochromes aa₃, b, and c in synchronous cultures grown on (A) glucose and (B) galactose media. Arrows designate new rounds of bud formation.

Fig. 7. Relationship between cytochrome c oxidase activity and cytochrome aa₃ concentration during synchronous growth on galactose media. Similar relationships were observed in glucose-grown cells. Arrows designate new rounds of bud formation.

* S. F. Cottrell and M. Rabinowitz, unpublished data.
FIG. 8. Effect of addition of chloramphenicol (CAP) on cytochrome c synthesis in galactose-grown cells. A, B, C, and D show the results, respectively, of no chloramphenicol addition, chloramphenicol addition prior to, after 40%, and after 70%, of the first cell cycle. The unlabeled arrows designate new rounds of bud formation.

FIG. 9. Effect of addition of chloramphenicol (CAP) at different points in the cell cycle on cytochrome aa₃ synthesis in galactose-grown cells. A, B, C, and D show the results, respectively, of no chloramphenicol addition, chloramphenicol addition prior to, after 40%, and after 70% of the first cell cycle. The unlabeled arrows designate new rounds of bud formation.

FIG. 10. Effect of addition of chloramphenicol (CAP) on cytochrome b synthesis in galactose-grown cells. A, B, C, and D show the results, respectively, of no chloramphenicol addition, chloramphenicol addition prior to, after 40%, and after 70%, of the first cell cycle. The unlabeled arrows designate new rounds of bud formation.

scans indicated no increase in the calculated cytochrome b concentration even when the cytochrome c concentration was increased 10-fold although in this case the absorption maximum of cytochrome b was a barely discernible shoulder on the cytochrome c peak.

We also tried to evaluate whether continued cytochrome b accumulation represented a mitochondrial component or whether, after chloramphenicol administration, an extramitochondrial b type cytochrome having an absorption maximum at 558 to 559 nm accumulated instead. A synchronous culture was treated with 2.4 mg per ml chloramphenicol prior to the first round of bud formation. Small aliquots of cells taken during the next two cell cycles were processed and scanned according to the standard procedure. From other aliquots, sampled at the same time, mitochondria were isolated and purified from spheroplasts according to the method of Rabinowitz et al. (33). The ratios of the concentrations of cytochromes aa₃:b:c derived from liquid nitrogen temperature scans of whole cells and of washed mitochondria were the same (Table I). It seems probable that the cytochrome b absorption band measured in whole-cell preparations reflects the changes in mitochondrial cytochrome b concentration.

The possibility that our observation is strain specific is not likely since we observed similar patterns of cytochrome b synthesis in the presence of chloramphenicol in three other strains of Saccharomyces cerevisiae.

DISCUSSION

Our data clearly show that the synthesis of cytochromes of the inner mitochondrial membrane is continuous throughout several synchronous cell cycles in yeast. We cannot differentiate, however, whether the accumulation rate increases exponentially
could be accounted for. Not only was there a temporal dissociation between the increments in cytochrome \( a_d \) and cytochrome oxidase but also a quantitative dissociation. Whereas the oxidase approximately doubles in each succeeding cell cycle this is not true for cytochrome \( a_d \), which does not show such substantial increments except in the second cycle of cells growing in galactose. The meaning of this dissociation is not clear, but it does seem to indicate that under certain circumstances the cytochrome \( a_d \) may be present in excess of its functional association with active oxidase complexes.

The capacity of chloramphenicol applied at any time of the synchronous growth cycle to inhibit cytochrome \( a_d \) synthesis without a lag supports the conclusion that at least some components of the enzyme complex are synthesized continuously throughout the cell cycle of synchronous cultures. As chloramphenicol selectively inhibits the synthesis of polyopeptides by mitochondrial ribosomes (13–15), it may be concluded that the synthesis of mitochondrial products necessary for the formation of cytochrome \( a_d \) is carried on throughout the cycle of synchronous cells, and that no significant pool of these products accumulates. The continuous synthesis of cytochrome \( a_d \) polyopeptide on mitochondrial ribosomes throughout the yeast cell cycle differs from data for mammalian cells in which in vitro mitochondrial polyopeptide synthesis occurs primarily in the G1 and G2 periods (2).

In contrast to the results which we obtained for cytochrome \( a_d \), the effect of chloramphenicol on the absorbance of cytochrome \( b \) is not readily interpreted. That we were in fact measuring respiratory chain cytochrome \( b \) is strongly confirmed by the finding of a similar ratio of cytochromes \( a_d:b:c \) in whole cell and in isolated mitochondrial preparations from chloramphenicol-inhibited cultures. Intramitochondrial synthesis of a cytochrome \( b \) component has been reported in Neurospora cells by Weiss (43, 44), and long term exposure of yeast to chloramphenicol results in the apparent loss of both cytochromes \( a_d \) and \( b \) (14, 31, 32). Our short term inhibition studies limited to 2 or 3 generations, however, indicate only a transient inhibition of cytochrome \( b \) synthesis by chloramphenicol. In a short term study of asynchronous lactate-grown yeast, Mahler and Perlman (26) also observed the continued formation of some cytochrome \( b \) after the addition of sufficient chloramphenicol to inhibit cytochrome \( a_d \) synthesis completely. Possibly the sustained inhibition of cytochrome \( b \) synthesis appears only after long term exposure to chloramphenicol because there is a large pool of mitochondrial synthesized cytochrome \( b \) peptide. A slightly diminished rate of synthesis (not detectable in the present study) would also account for the long term effect of the drug on cytochrome \( b \) reported by others. Alternatively, cytochrome \( b \) may continue to be synthesized in the presence of chloramphenicol, but in long term studies its \( a \) absorption peak may be masked by a greatly enhanced concentration of cytochrome \( c \). Such an enhancement in the rate of cytochrome \( c \) accumulation in the presence of chloramphenicol has been observed by us and reported by others (26, 45). It is doubtful that this accounts for the reported observations since we were able to measure the absorbance of cytochrome \( b \) even in the presence of a large excess of cytochrome \( c \). Even after cytochrome \( c \) had been removed by salt extraction, no cytochrome \( b \) was detected in subcellular fractions derived from cells exposed to chloramphenicol for extended periods (31).

We can offer no simple explanation for the observation that the transient inhibition of cytochrome \( b \) accumulation was immediate.

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2 S. F. Cottrell and M. Rabinowitz, unpublished data.

3 L. King and G. S. Getzl, unpublished data.

4 S. F. Cottrell, G. S. Getzl, and M. Rabinowitz, unpublished data.
cytochrome accumulation will be found to be generally applicable.

Our results indicate that, at least as reflected in the pattern of cytochrome accumulation during synchronous growth, mitochondrial membrane formation occurs throughout the cell cycle by a process of continual accretion of new components. The possibility has to be considered that this pattern of cytochrome accumulation is peculiar to the experimental circumstances used and might not apply to cells growing exponentially that have not been exposed to cycles of feeding and starvation. However, the cells grown synchronously in both glucose and galactose exhibited good synchrony with a discrete doubling of cell number, DNA, and in the case of galactose cells, in cytochrome oxidase activity. They thus exhibited balanced growth with respect to these parameters. The technique of synchronization employed made it necessary to initiate synchronous growth with cells grown to stationary phase in glucose which was subjected to alternate feeding and starvation. The cells were therefore partially or completely derepressed. During the course of synchronous growth, mitochondrial membrane formation occurs throughout the cell cycle but was delayed when added prior to initial budding.

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