Synthesis and Assembly of Cytochrome *c* Oxidase in Synchronous Cultures of Yeast†

T. Somasundaram and J. Jayaraman*

ABSTRACT: Yeast cells growing synchronously in glucose medium accumulate in the cytosol, the cytosolically made subunits of cytochrome oxidase, during the G_1 and early-S phases. The mitochondrially made subunits, on the other hand, are detected only after the mid-S phase. The cytosolically synthesized subunits are integrated into the membrane after the mid-S phase.

The enzyme cytochrome *c* oxidase (EC 1.9.3.1) is a supramolecular complex consisting of seven polypeptide subunits, heme *a*, copper, and phospholipids (Weiss et al., 1971; Mason et al., 1973; Rubin & Tzagoloff, 1973a; Sebald et al., 1973). The biogenesis and assembly of such a complex involve close cooperation of the two genetic systems (Weiss et al., 1971; Sebald et al., 1972; Mason & Schatz, 1973; Rubin & Tzagoloff, 1973b). The topography of the various polypeptides in the enzyme has been well studied by different approaches such as the use of subunit-specific antisera (Poyton & Schatz, 1975a; Hackenbrock & Hammon, 1975), surface labeling with covalent probes (Eytan & Schatz, 1975), and chemical analysis of subunit structure (Sebald et al., 1973; Poyton & Schatz, 1975b). These studies have established that the cytochrome oxidase in yeast is made up of three hydrophobic polypeptides synthesized in mitochondria and the other four subunits (of which three are hydrophilic) are derived from the cytoplasm. The hydrophilic subunits are distributed asymmetrically on both sides of the membranes (Eytan et al., 1975) whereas the hydrophobic subunits are partially or completely buried within the lipid bilayer of the inner mitochondrial membrane (Sebald et al., 1973; Poyton & Schatz, 1975b).

With our interest on the biogenesis of mitochondrial enzymes (Jayaraman et al., 1966, 1974; Chandrasekaran & Jayaraman, 1978), we have investigated the various aspects of biogenesis in synchronous cultures of yeast, and the results are presented here. The advantages of using synchronous cultures have already been discussed (Somasundaram & Jayaraman, 1979), and the biosynthesis of the ATPase complex in such systems has been described in the following paper in this issue (Somasundaram & Jayaraman 1981). In the case of ATPase, an allotopic enzyme, the catalytic activity could be measured both in the cytosol, where it is synthesized but remains in an unintegrated form, and in the mitochondrial membranes (Senior, 1973; Tzagoloff et al., 1973). However, the expression of cytochrome oxidase activity requires the presence of all subunits in the membrane; therefore, one has to rely extensively on immunoprecipitation and gel electrophoretic techniques to identify the subunits of different origin.

Materials and Methods

Methods for the synchronous growth, isolation of mitochondria, labeling conditions, electrophoresis, and analysis of the radioactive profile in the gel are as given in the following paper (Somasundaram & Jayaraman, 1981). estimation of protein was followed by the method of Lowry et al. (1951).

Cytochrome c Oxidase. Cyt *c*[1] oxidase was assayed following the procedure of Wharton & Tzagoloff (1967). The reaction mixture contained 30 μmol of phosphate buffer (pH 7.0) and 20 μmol of cytochrome *c* in a total volume of 3 mL. Cyt *c* dissolved in 0.1 M phosphate buffer was reduced with ascorbate, and the excess ascorbate was removed by dialysis against 0.1 M phosphate buffer for 18 h at 0–5 °C with three to four changes of buffer. The activity of the enzyme was measured by following the decrease in the absorbance at 550 nm in a Beckman DU 2 spectrophotometer for 2 min. The first-order rate constant was calculated according to Smith (1965). The enzyme activity was calculated by using the millimolar extinction coefficient of 19.2 at 550 nm for cyt *c*.

Immunoprecipitation of Cytochrome c Oxidase. The procedure of Ebner et al. (1973) was followed. All steps were performed at 4 °C. To a suspension of mitochondria having 0.6 M mannitol and 2 mM EDTA, pH 7.4, were added sufficient KCl and a 20% sodium deoxycholate solution (pH 7.0) to give a final concentration of 3 mg of sodium deoxycholate per mg of mitochondrial protein and 1.2 M KCl. This mixture was gently stirred for 8 h and then centrifuged for 30 min at 2000*g*.

The supernatant which contains 60% of mitochondrial proteins was treated with antisera against holoenzyme and also with antisera against individual subunits IV, V, and VI. For the holoenzyme, the procedure described by Chandrasekaran & Jayaraman (1978) was used, and for the subunit-specific precipitation, the procedure of Ebner et al. (1973) was followed. The immunoprecipitates were washed twice with 10 mM Tris-HCl buffer, pH 8.2.

Results

Cytochrome Oxidase Activity during Cell Cycle. Cytochrome oxidase activities of the mitochondrial pellets isolated during the different stages of growth in a synchronously growing population of yeast are shown in Figure 1. The characteristics of the synchronous cultures have been described by Somasundaram & Jayaraman (1979). It can be seen that there is a peak of activity at the mid-G_2 phase, pattern simulating that of respiration and ATPase, as reported earlier.

Synthesis of Subunits in Cytosol. Cells were exposed to ^{14}C-labeled chlorella hydrolysate (0.5 μCi/mL) during the

† From the Department of Biochemistry, School of Biological Sciences, Madurai Kamaraj University, Madurai 625021, India. *Received March 24, 1980; revised manuscript received January 28, 1981.* This research project was supported by a grant from the Indian National Science Academy.

[1] Abbreviations used: Cap, chloramphenicol; cyt *c*, cytochrome *c*; PMS, postmitochondrial supernatant; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; CHI, cycloheximide.

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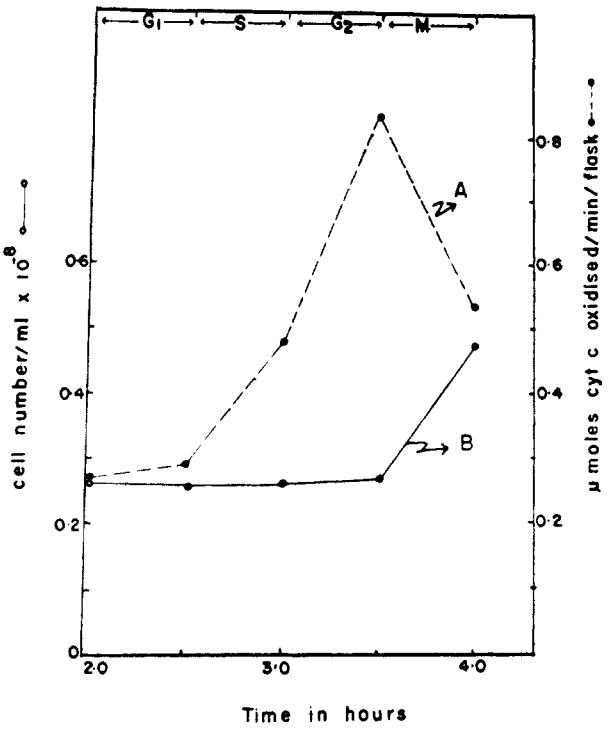

FIGURE 1: Cytochrome oxidase activity at various phases of the cell cycle. Enzyme activity is expressed as μmol of cyt *c* oxidized min^{-1} $cell^{-1}$ from 50 mL of culture. Curve A represents activity of cyt *c* oxidase, and curve B represents growth.

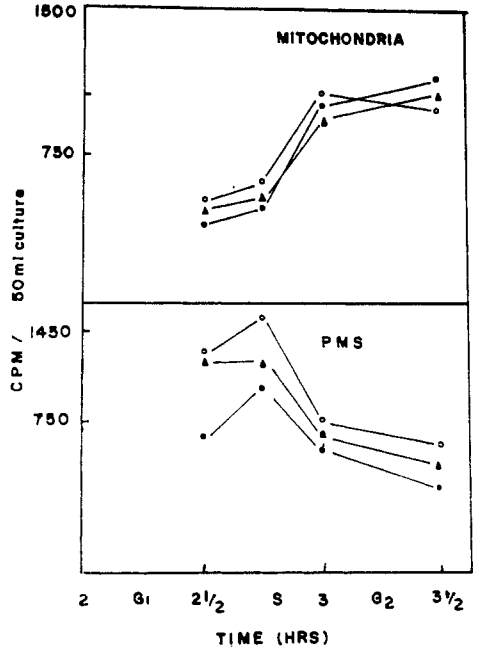

FIGURE 2: Synthesis of cytosolic subunits. Experimental details as given in the text. (○) Subunit IV, (▲) subunit V, and (●) subunit VI.

G_1 phase for 20 min. The cells were then transferred to cold used media containing Cap. At various time intervals, the cells were harvested and mitochondria isolated. Both the mitochondrial and postmitochondrial supernatant fractions were treated with individual antisera against subunits IV, V, and VI. The immunoprecipitates thus obtained were washed, and the amount of radioactivity was measured. The results are given in Figure 2.

All three subunits studied show similar patterns. In the mitochondrial fractions, the radioactivity in the immunoprecipitates remains low till the mid-S phase but within 15 min

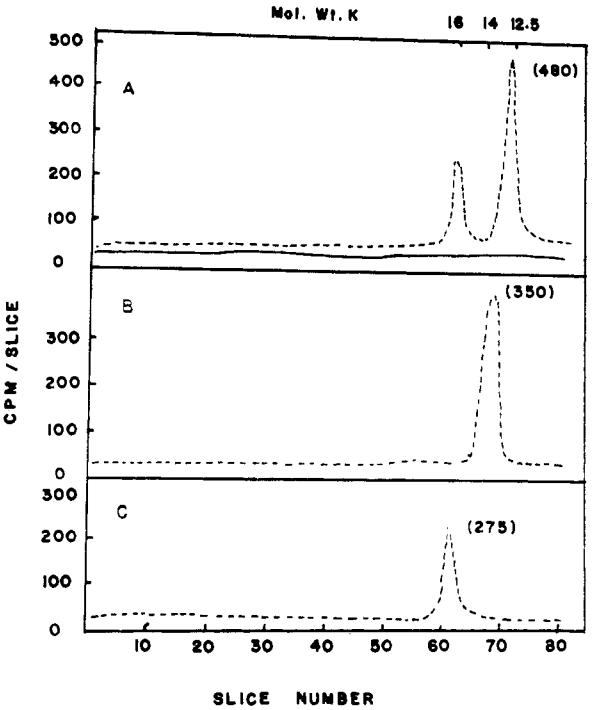

FIGURE 3: Radioactive profiles of immunoprecipitates from PMS. Experimental details are given in the text. The numbers in parentheses represent the total counts in the peak. Panels A, B, and C represent subunits VI, V, and IV, respectively.

thereafter shows a sharp increase. This higher level is maintained till the end of the G_2 phase. In the postmitochondrial supernatant, the pattern is almost inverse. Up to the mid-S phase, the radioactivity content is high. In fact, counts in subunits IV and VI even increase slightly, although the cells were in cold medium. But immediately after the mid-S phase, they decrease rapidly until the end of the G_2 phase. These results indicate that the cytosolically synthesized subunits IV, V, and VI are accumulated in the cytosol till the mid-S phase and only after this phase are they transported to the mitochondria.

Identity of Cytosolically Synthesized Subunits. Cells were labeled with ^{14}C-labeled algal hydrolysate during the G_1 phase and transferred to cold medium as before. At the mid-S phase cells were harvested, and postmitochondrial supernatant was obtained and immunoprecipitated with the three antisera as above. The immunoprecipitates were analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis. The radioactive profiles are shown in Figure 3.

The subunit-specific antisera could precipitate only the corresponding subunits, with molecular weights 16 × 10^3, 14 × 10^3, and 12.5 × 10^3, respectively, for subunits IV, V,and VI (Buse & Steffens, 1976; Lewin et al., 1980). The radioactive profiles of the gel pattern show these subunits to be synthesized individually. Only in the case of subunit VI is a small peak at 16 × 10^3 daltons also seen. At the moment, the identity of this peak is unknown. But the results definitely rule out the possibility that the cytoplasmic subunits are synthesized as a polyprotein precursor as claimed by Poyton & McKemmie (1979a,b).

In an earlier report (Jayaraman & Somasundaram, 1981) where we used antisera for the holoenzyme, under similar experimental conditions, we observed a radioactive peak at M_r 58 × 10^3, which corresponded to the precursor protein reported by Poyton & McKemmie (1979a,b). Recently it has been shown, however, that this M_r 58 × 10^3 peak could be a contaminant rather than the bona fide precursor of the subunits

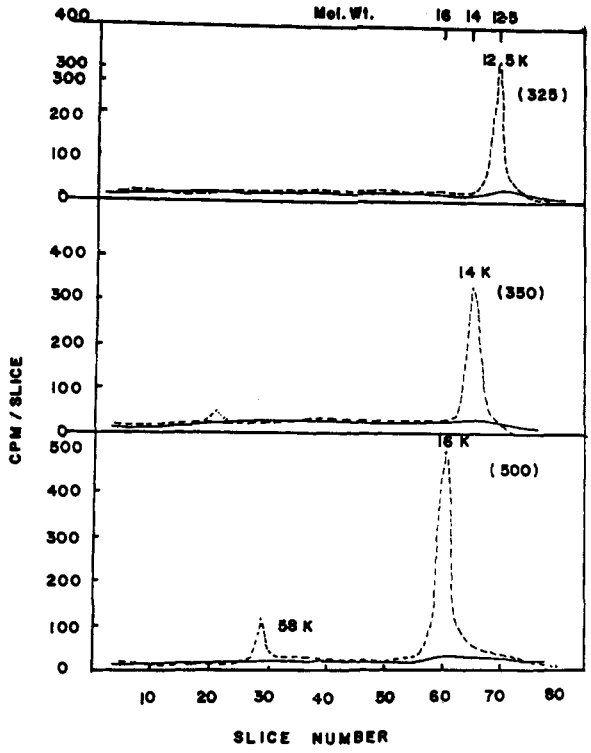

FIGURE 4: Radioactive profile of immunoprecipitate from late-G_1 phase and late-S phase mitochondria. Experimental conditions as for Figure 3. Numbers in parentheses refer to the total counts in the peak, and the numbers 12.5K, 14K, and 16K indicate the molecular weight of subunits VI, V, and IV, respectively. (—) late G_1 and (- - -) late S.

(Lewin et al., 1980; our present data).

Cytosolic Subunits in Mitochondria. Another set of experiments were carried out under similar conditions. Mitochondria were isolated at the end of G_1 and S phases. They were treated with antisera as above and the immunoprecipitates analyzed. The G_1 phase mitochondria showed no radioactive peaks. The radioactive profiles of immunoprecipitates for the three subunits obtained from the late S phase are shown in Figure 4. Except for a small peak at M_r 58 × 10^3 in the immunoprecipitate of subunit which we tend to discount as a contaminant, the three subunits have been immunoprecipitated as individual entities. Lewin et al. (1980) have reported that these subunits are synthesized individually, but in precursor forms, and then transported to mitochondria. Under our conditions, however, we do not find evidence for such individual precursors. The possibility exists that the accumulated precursors could have been digested by a metalloprotease released from the matrix during grinding and which is apparently specific to mitochondrial proteins (G. Schatz, personal communication; J. Ashraf, unpublished observations).

Synthesis of Mitochondrial Subunits. The next series of experiments was designed to study the biosynthesis of mitochondrially made subunits. The cells at different phases of growth were exposed to ^{14}C-labeled chlorella hydrolysate for 15 min (0.2 μCi/mL) in the presence of CHI (100 μg/mL). Immediately after this, cells were harvested, the immunoprecipitates of the mitochndrial pellet were obtained, and the total radioactivity was determined. For immunoprecipitation, antiserum against whole enzyme complex was used.

As can be seen from Table I, there is a sudden increase of 3–4-fold in the immunoprecipitable radioactivity of mitochondria between 2.75 and 3.0 h, which is the late-S phase.

Another set of similar experiments was carried out. The experimental details are given in the legend to Figure 5. The radioactive profile of the immunoprecipitates after polyacrylamide gel electrophoresis is given in Figure 5.

Table I: Time of Synthesis of Mitochondrial Subunits of Cytochrome *c* Oxidase

time (h)	phase	total immunoprecipitable counts
2.00–2.25	early G_1	170
2.25–2.5	late G_1	170
2.5–2.75	early S	140
2.75–3.00	late S	270
3.00–3.25	early G_2	600

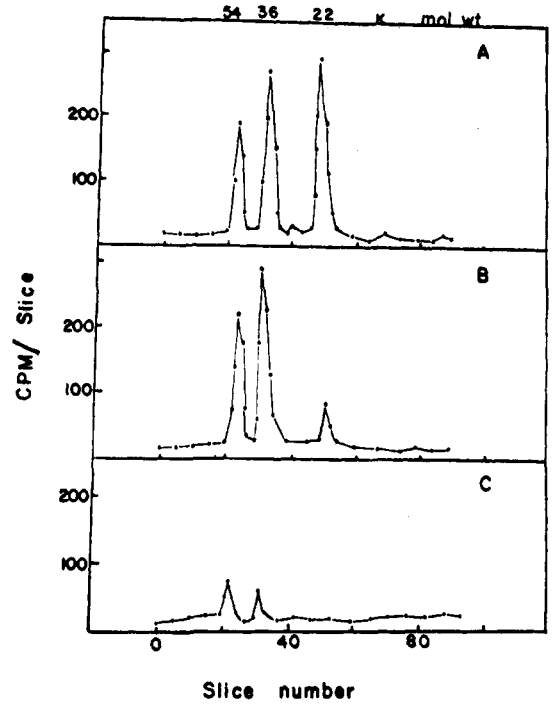

FIGURE 5: The cells were labeled with ^{14}C-labeled chlorella hydrolysate (0.8 μCi/mL) in the presence of CHI between 2.5 and 2.75 (early-S phase), 2.75 and 3.00 (late-S phase), and 3.00 and 3.25 h (early-G_2 phase). After labeling, the cells were harvested. Cyt oxidase was immunoprecipitated from mitochondrial fractions and analyzed in $NaDodSO_4$–polyacrylamide gel electrophoresis. Panels A, B, and C represent the analysis of the immunoprecipitates from the cells labeled early-G_2, late-S, and early-S phases, respectively.

The labeling pattern of the mid-S phase cells (Figure 5, panel C) barely shows the presence of two peaks with molecular weights of 54 × 10^3 and 36 × 10^3, which correspond to subunits I and II of cytochrome oxidase (Chandrasekaran et al., 1980). By the early-G_2 phase (Figure 5, panel B), the labeling in these two peaks has become prominent, and another peak with a molecular weight of 22 × 10^3, corresponding to subunit III, gets labeled. All three peaks are significantly labeled by the mid-G_2 phase (Figure 5, panel A).

The above results clearly indicate that (a) the mitochondrial subunits are made available only during the early-G_2 phase and (b) the synthesis of mitochondrial subunits follows a temporal sequence.

Discussion

In the following paper (Somasundaram & Jayaraman, 1981), we have shown that during the biogenesis of the ATPase complex, the cytosolic and mitochondrial syntheses of the constituent components take place during different phases of the cell cycle. Results presented in this paper also point to a similar situation in the synthesis of cytochrome oxidase. Cottrell et al. (1975) reported that there was continuous synthesis of cytochromes during the cell cycle of *Saccharo-*

myces cerevisiae whereas cytochrome oxidase was expressed only during the late-S phase, which correlated with the synthesis of cardiolipin. In the present study, we have analyzed the synthesis and assembly of the individual subunits.

The subunits synthesized by the cytosol are present until the mid-S phase, but their mitochondrial counterparts, which are needed for the expression of the enzyme activity, are made available only during the G_2 phase. These conclusions are based on immunoprecipitation combined with $NaDodSO_4$–polyacrylamide gel electrophoresis techniques.

The cytoplasmically synthesized subunits are made as individual polypeptides and not as the polyprotein precursor, as suggested by Poyton & McKemmie (1979a,b). This is confirmed by the use of individual subunit-specific antisera. These individual subunits accumulate in the cytosol until the mid-S phase and only then are transported to the mitochondria.

The mitochondrially made subunits show a temporal sequence in their synthesis, subunits I, II, and III in that order. Similar sequential appearance of these subunits has been shown in derepressing spheroplasts of yeast (Chandrasekaran et al., 1980) and also during germination of ascospores (Brambl & Handschin, 1976).

The time of synthesis of mitochondrial components has been mainly assessed by pulse labeling in the presence of CHI. Incorporation of radioactive label into a particular peptide or subunit depends not only on the time of synthesis but also on the pool size of the precursors for these components, if any. If there is a large pool size of the precursor present, the component under study may not show an incorporation until all the precursor molecules are converted into the particular subunit. The difference in pool sizes of the precursor polypeptides of cytochrome oxidase subunits has been reported in *Neurospora crassa* (Schwab et al., 1972). The difference in the pool sizes has been attributed to the difference in the half-lives of the precursor polypeptides. Further studies on the precursor and their half-lives for these mitochondrially made components will indicate the exact times of synthesis of these products. Moreover, one has to distinguish between the time of assembly and the time of synthesis, especially with membrane-bound enzymes or proteins.

The important point is that in whatever form the cytosolically made proteins is transported from the cytosol to the membrane, their integration must await the synthesis of mitochodnrial counterparts.

The question of what triggers mitochondrial protein synthesis at the particular point in the cell cycle is still open. Stimulation of mitochondrial protein synthesis by cytosolic products is well established (Lin et al., 1974; Ibrahim & Beattie, 1976). The work of Poyton & McKemmie (1976) suggests that the cytoplasmic subunits could trigger the mitochondrial synthesis of cytochrome oxidase. Ono et al. (1975) have reported that a nuclear mutant lacks subunit III of mitochondrial origin. Recently Ohashi & Schatz (1980) have claimed that GDP is the trigger molecule. Our own results show that cAMP stimulates mitochondrial protein synthesis (Chandrasekaran & Jayaraman, 1978). The experimental system described above opens up avenues to dissect out these events.

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Synthesis and Assembly of Adenosinetriphosphatase in Synchronous Cultures of Yeast†

T. Somasundaram and J. Jayaraman*

ABSTRACT: Maximal respiration and expression of mitochondrial enzymes are found at the late-S phase of yeast cells growing synchronously in glucose medium. Adenosinetriphosphatase (ATPase) activity follows a similar pattern. However, the cytosolically synthesized F_1-ATPase and also that released from the membrane accumulate in the cytosol during the G_1 and early-S phases. After the mid-S phase, when the mitochondrially synthesized membrane factors are available, the enzyme migrates to the membrane and is integrated.

The process of mitochondrial biogenesis has been shown to involve two genetic systems, namely, the nuclear and mitochondrial (Ashwell & Work, 1970; Linnane et al., 1972; Schatz & Mason, 1974; Freedman & Chan, 1978). A vast majority of the mitochondrial proteins are synthesized on the cytoplasmic ribosomes and transported to mitochondria (Beattie, 1971). The interrelationship between the two systems has been conveniently studied in yeast by the use of differentially acting antibiotics, Cap[1] and CHI, which inhibit the mitochondrial and the cytoplasmic protein syntheses, respectively. The role of mitochondrial protein synthesis during mitochondrial membrane formation has been shown in the case of cyt *c* oxidase, ATPase, and $CoQH_2$–cyt-*c* reductase. However, most of these studies have been carried out under rather nonphysiological conditions, namely in the presence of protein synthetic inhibitors (Schatz & Mason, 1974; Chandrasekaran et al., 1980).

In an earlier work, Smith et al. (1968) showed that in synchronous cultures of *Saccharomyces lactis*, the mitochondrial DNA synthesis is out of phase with that of nuclear DNA during the cell cycle. In our laboratory, we had shown that the respiratory pattern of the cells growing synchronously gave maximum respiration at the G_2 phase (Dharmalingam & Jayaraman, 1973). It was thus of interest to study whether synchronous cultures could be used as "physiological" situations to understand the mechanisms of mitochondrial assembly. Preliminary evidence showed that in such cultures electron-transport enzymes also show maximum activity during G_2 phases (Jayaraman et al., 1975; Somasundaram & Jayaraman, 1979). In this paper, we discuss the synthesis of ATPase enzyme and its incorporation into the membrane.

Materials and Methods

Inhibitors. (a) Cycloheximide (CHI) was a kind gift of Dr. H. O. Halvorson, MA, and Dr. G. Schatz, Biocenter, Basel, Switzerland. (b) Chloramphenicol (Cap) was purchased commercially from Parke Davis, India. (c) Oligomycin was a gift of Dr. Somlo, Gif-Sur-Yvette, France.

Radioactive Chemical. ^{14}C-Labeled chlorella hydrolysate (specific activity 42 mCi/mmol of carbon), purchased from the Isotope Division, Bhabha Atomic Research Centre, Bombay, India, was used for amino acid incorporation studies.

Organism Used. The organism used in this study was *Saccharomyces cerevisiae* NCIM 3095, obtained from the National Collection of Industrial Microorganisms, National Chemical Laboratory, Poona, India. It is a diploid strain. This strain has been used in this laboratory for the past several years.

Maintenance. The cultures were maintained in nutrient agar slants containing 3% malt extract, 1% glucose, and 2% agar and stored at 0–4 °C. Subculturing was done once every 10 days.

Growth Media. The following were the media used for synchronization and growth. (*a*) *Wickerham's Double-Strength Medium.* This medium contained 2% glucose, 0.6% Difco yeast extract, 0.6% Difco malt extract, and 1% oxoid mycological peptone. (*b*) *Wickerham's Single-Strength Medium.* This medium was prepared by diluting the double-strength medium with equal volume of sterile water. (*c*) *Starvation Medium.* This medium contained 0.075% KCl, 0.025% $CaCl_2$ and 0.05% $MgCl_2$. (*d*) *Growth Medium.* For routine experiments on synchronous growth, synchronized cells were grown in the medium having the following composition: 1% glucose, 0.4% yeast extract, 0.9% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.04% $CaCl_2$, and 0.04% $(NH_4)_2SO_4$. Throughout the studies, 250-mL conical flasks containing 50 mL of medium were used. (*e*) *Used Medium.* In some experiments ^{14}C labeling was done at the desired phase, and cells were then transferred to nonradioactive medium. In these cases, the "used medium" was utilized. This medium was obtained from flasks containing control cells which had undergone the same physical treatment as others, except without radioactive material. At appropriate time intervals, the cells were centrifuged, and the supernatant of the medium was used as the "used medium".

† From the Department of Biochemistry, School of Biological Sciences, Madurai Kamaraj University, Madurai 625021, India. *Received March 24, 1980; revised manuscript received January 28, 1981.* This research project was supported by a grant from the Indian National Science Academy.

[1] Abbreviations used: ATPase, adenosinetriphosphatase; Cap, chloramphenicol; CHI, cycloheximide; cpm, counts per minute; PMS, post-mitochondrial supernatant; PRS, postribosomal supernatant; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3CCOOH, trichloroacetic acid; cyt *c*, cytochrome *c*; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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