Somasundaram, T., & Jayaraman, J. (1981) *Biochemistry* (following paper in this issue).
Tzagoloff, A., Rubin, M. S., & Sierra, M. F. (1973) *Biochim. Biophys. Acta 301*, 71.
Weiss, H., Sebald, W., & Bucher, Th. (1971) *Eur. J. Biochem. 22*, 19.
Wharton, D. C., & Tzagoloff, A. (1967) *Methods Enzymol. 10*, 245.

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.

0006-2960/81/0420-5373$01.25/0

Induction of Synchrony. The procedure of Williamson & Scopes (1962) was used with minor modifications. Yeast cells were grown at 25 °C in a rotary shaker for 10 days in Wickerham's double-strength medium. The cells were then collected by centrifugation (2000*g* for 3 min), washed twice with sterile saline, and suspended in a 20% aqueous solution of sorbitol at a concentration of 6 mg wet weight/mL. This suspension was centrifuged in swing-out buckets at 600*g* for 6 min. The sediment was resuspended in 20% sorbitol and recentrifuged as mentioned above, and the procedure was repeated until the deposit was found to be free of smaller cells on microscopic observation. The pellet was suspended in 50-mL amounts of sterile single-strength Wickerham's medium (5–8 mg wet weight/mL) and kept at 0–5 °C for 18 h. By this time, the cells settled down, and the clear supernatant was carefully decanted. The cells were then washed with sterile distilled water and then suspended in 100-mL amounts of starvation medium at a concentration of 4–5 mg wet weight/mL. The flasks were kept in a shaker at 25 °C for 6 h, after which the cells were harvested, again suspended in Wickerham's single-strength medium, and left at 0–5 °C for 18 h. This feeding starvation cycle was repeated twice, normally with three feedings and two starvations.

Growth. Growth was monitored by visual counting of the number of cells with a hemocytometer as described by Williamson (1964).

Oxygen Uptake. Oxygen uptake was measured by using a Clark-type electrode (YSI 4004, Yellow Spring, Ohio) attached to a recorder. Washed cells (20 mg wet weight) were added to the reaction vessel containing 200 μmol of phosphate buffer (pH 7.0) and 200 μmol of glucose in a total volume of 3 mL. The measurements were made at 30 °C, and the oxygen concentration of the reaction medium was assumed to be 240 nmol of O_2/mL (Lessler, 1969).

Isolation of Mitochondria. The procedure described by Jayaraman et al. (1966) was followed. The cells at times indicated were harvested and washed once with sterile saline. To the packed cells was added an equal amount of a mixture containing Carborundum and Celite (1:1 w/w). The contents were mixed and ground in a cold mortar for 30 s. The resulting paste was extracted with 10 mM Tris-HCl buffer in 0.25 M sucrose, pH 8.3 (12 mL/g wet weight of cells). The extract was centrifuged at 600*g* for 10 min to sediment the cell debris, nuclei, etc. The supernatant was recentrifuged at 10000*g* for 20 min. The resulting pellet was washed twice with the same buffer and used as the source of mitochondria. All operations were carried out in a Janetzki refrigerated centrifuge, Model K-24. The integrity of the mitochondrial preparation was checked by the ADP/O ratio and respiratory control. The mitochondrial preparations showed a respiratory control index of 2.5 with an ADP/O ratio of 1.7, with succinate as substrate. Also the estimation of heme *c* in both the mitochondria and total cellular homogenate showed 80% recovery in the mitochondrial fraction.

ATPase. ATPase was assayed following the procedure of Tzagoloff (1970) with minor modifications. The enzyme activity was monitored at 30 °C for 10 min in a total volume of 1 mL. The reaction mixture contained 50 mM Tris-HCl (pH 8.5), 4 mM ATP, 5 mM $MgSO_4$, and 50–60 μg of mitochondrial protein as the enzyme source. For the assay of ATPase in the postribosomal supernatant, 100 μg of supernatant protein was added. The reaction was initiated by adding ATP, stopped by the addition of 0.5 mL of 10% Cl_3-CCOOH, and kept at 0 °C for 15 min. The protein precipitate was centrifuged at 3000 rpm for 10 min. The supernatant was assayed for P_i by the method of Fiske & Subbarow (1925). The total activity of ATPase is expressed as micromoles of P_i liberated per minute per cell from 50 mL of culture. In cases where oligomycin was used, it was added to a final concentration of 5 μg/mg of protein. In assays where antiserum was used, the enzyme preparation was incubated with the antiserum (in the ratio of 0.05 mL of serum to 150 μg of protein) for 45 min at 30 °C and then added to the reaction mixture.

Estimation of Protein. Protein estimation in all the samples was done following the method of Lowry et al. (1951). Bovine serum albumin was used as the standard.

Immunoprecipitation of ATPase. The method of Tzagoloff & Meagher (1971) was followed to immunoprecipitate the ATPase from mitochondrial preparation. Mitochondria were treated with Triton X-100 at a final concentration of 0.1% and centrifuged at 20000*g* for 15 min. The supernatant was treated with antiserum for ATPase enzyme. After incubation at 30 °C for 1 h, it was centrifuged at 7000*g* for 10 min. The precipitate was washed with Tris-HCl (10 mM, pH 8.3) buffer. A titration of Triton X-100 extract with antisera was carried out. From this, the optimal ratio of the antisera to mitochondrial protein was calculated to be 0.05 mL of antisera for 150 μg of mitochondrial protein and 0.1 mL of antiserum for 150 μg of postmitochondrial supernatant (PMS). The α subunit of ATPase was immunoprecipitated following the procedure described by Maccecchini et al. (1979).

Gel Electrophoresis. The $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out following the method of Weber & Osborn (1969). Acrylamide gel (10%) was used throughout. The gels were prepared by mixing 1 g of acrylamide and 27 mg of bis(acrylamide) with 5 mL of gel buffer and 5 mL of distilled H_2O. The solution was degassed at room temperature in vacuo for about 20 s. *N,N,N′,N′*-Tetramethylethylenediamine (0.005 mL) and 7–8 mg of ammonium persulfate were added. The solution was mixed and pipetted into vertical glass tubes of 6-mm diameter with their bases covered with rubber rings. Water was carefully layered over the solution to ensure a flat gel surface.

Electrophoresis was carried out at room temperature (about 30 °C). Samples containing 200 μg of protein were layered over the gels, and electrophoresis was carried out at 5 mA/gel for 18 h. The gels were stained with amido black (0.2% methanol–acetic acid–water, 4:4:1) and destained with 7.5% acetic acid (67.5 mL of water, 25 mL of 50% methanol, and 7.5 mL of acetic acid).

Radioactive Profile in Gels. After electrophoresis, the gels were sliced to 1.2–1.3-mm-thickness slabs with a manual gel slicer. Slight deviations of peak positions are due to this. The slices were solubilized with 0.5 mL of hydrogen peroxide for 3 h at 80 °C. Aliquots (200 μL) from this were placed on Whatman No. 3 disks and dried. After immersion of the disks in 5 mL of scintillation fluid (liquid Scintillator NE 213, Nuclear Enterprises Ltd., Edinburgh), the radioactivity was counted at 80% efficiency for ^{14}C in a liquid scintillation counter.

Results

Respiration and ATPase Activity during Synchronous Growth. Figure 1 shows the synchronous growth of the yeast cell under our experimental conditions. Normally four cycles could be repeated. The doubling ratio was 1.8, acceptable for synchronous cultures (Mitchison, 1971). Following the method of Hartwell (1974), based on the morphological observations of bud formation, position of the nuclei, total DNA content, and [^{3}H]uridine incorporation into DNA, different phases have been designated. The G_1 phase is the time duration of an

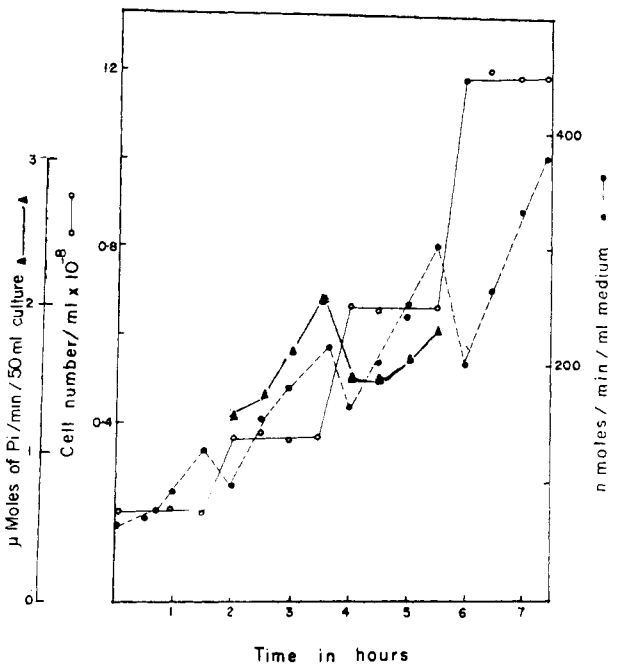

FIGURE 1: Synchronized cells were inoculated into normal growth medium, and the growth and O_2 uptake were followed. ATPase activity of the whole cell homogenate is given for the second division of cycle alone. (○) growth; (●) oxygen uptake as nmol min^{-1} (mL of medium)$^{-1}$; (▲) total ATPase activity/cell from 50 mL of culture.

unbudded cell giving rise to a bud (bud emergence). The S phase is the period of DNA synthesis. The G_2 phase represents the period after DNA synthesis until the migration of the nucleus to the neck of the cell, where it undergoes the first phase of the nuclear division. This can be observed by the dumbbell shape of the nuclei. Under our conditions, all the phases occupy equal spans of time, an observation which coincides with that of Williamson (1964) and Elliott & McLaughlin (1978).

The oscillatory oxygen uptake pattern during the cell cycle has already been reported by Dharmalingam & Jayaraman (1973), and they ascribed it to the possible differential entry of glucose into the cells at various phases and the consequent repressive effect of the glucose or its catabolite on the mitochondria.

In Figure 1, the ATPase levels (of the total homogenates) are also given, but this is restricted to the second cycle alone. It is noteworthy that the behavior of ATPase simulates qualitatively the pattern of respiration. But quantitatively, it does not increase in content as does the respiration. Several hypothetical reasons can be given for this, but we contend by saying that we are dealing with whole homogenates of the cell. Later experiments show that in the membrane fractions the increase is 1.8–2.0-fold.

Preliminary experiments were also carried out to determine whether this ATPase activity measured was membrane bound or otherwise, using the criteria of oligomycin sensitivity and cold insensitivity (indicative of the membrane-bound state). The results are given in Figure 2. It is clear that only during the late-S phase and G_2 phase does the enzyme show maximum oligomycin sensitivity and cold insensitivity, suggesting maximal membrane binding. There are of course certain discrepancies between the two parameters, which are discussed later.

Intracellular Distribution. Yeast cells were harvested at different points during the cell cycle and fractionated, and intracellular distribution of the enzyme activity was studied. As can be seen from Figure 3A, there is an increase of the

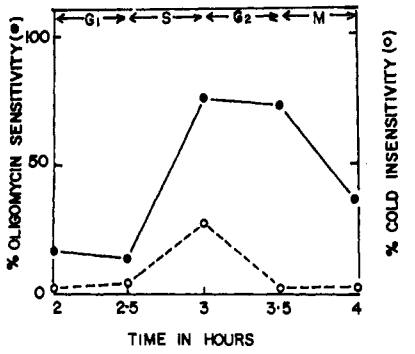

FIGURE 2: Oligomycin sensitivity and cold insensitivity of whole cell homogenates at different phases.

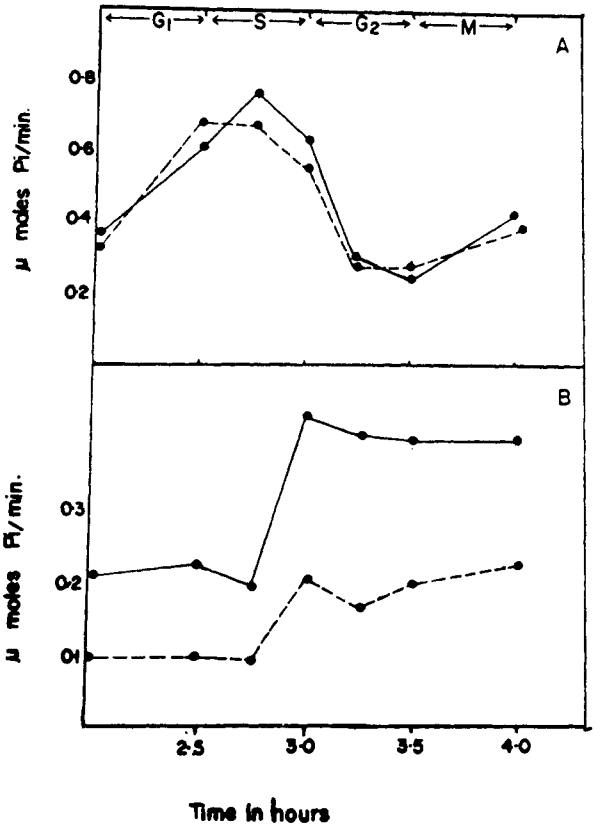

FIGURE 3: ATPase activities in mitochondrial and postribosomal supernatants at different stages. Panels A and B represent activities in the postribosomal supernatant and mitochondrial fractions, respectively. ATPase activity is expressed per cell from 50 mL of culture. (●) Activity in the absence of oligomycin; (○) activity in the presence of oligomycin.

activity in the postribosomal supernatant until the mid-S phase, and then there is a decrease. On the other hand, the mitochondrial activity remains at a low level until the mid-S phase. During the late-S phase, the mitochondrial activity increases, concomitant with the decrease in the cytosol (Figure 3B).

Radioactive Labeling and Immunoprecipitation Studies. A series of experiments was then carried out to label the ATPase enzyme complex with ^{14}C-labeled algal hydrolysate and then isolate the labeled complex by immunoprecipitation with a specific antibody.

(*a*) *Pulse Labeling.* A pulse-labeling experiment was carried out. The cells were exposed to ^{14}C-labeled algal hydrolysate during the G_1 phase, at the end of which they were transferred to cold medium. The cells were harvested at different time intervals, and analyses of the radioactivity in the mitochondrial and postmitochondrial supernatant immunoprecipitates were carried out as before. As can be seen in Figure 4A, up to the

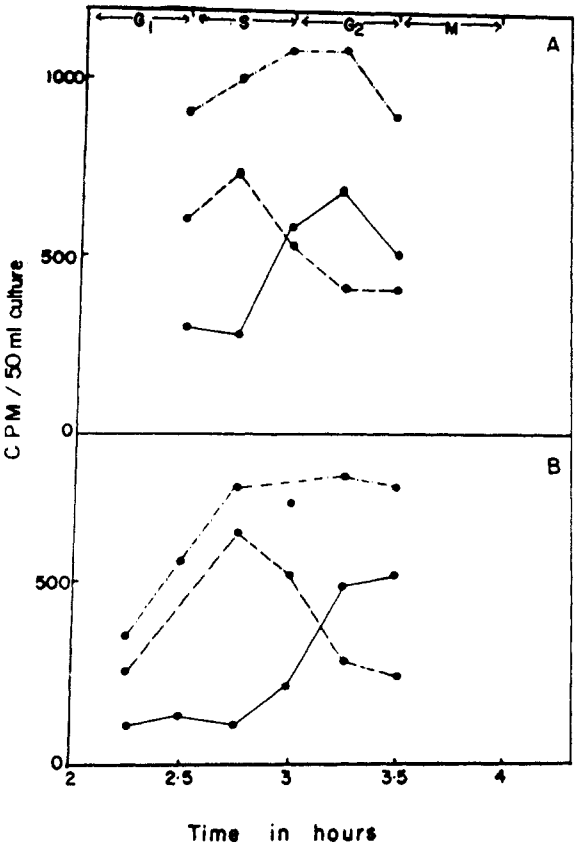

FIGURE 4: Analysis of ATPase by labeling and immunoprecipitation. Panel A shows the immunoprecipitable counts in the mitochondrial and postmitochondrial supernatant fractions during different phases after pulse labeling. Panel B shows the results when the cells were continuously labeled. (—) Mitochondrial fraction; (---) PMS fraction; (-·-) the added-up values of both mitochondrial and PMS fractions.

mid-S phase, the level of radioactivity in the PMS fraction was high and that of the mitochondrial fraction low. But just about the mid-S phase, the radioactivity in mitochondria increases, with a concomitant decrease in the PMS fraction.

(*b*) *Continuous Labeling.* The ^{14}C-labeled algal hydrolysate (0.5 μCi/mL of medium) was added to the synchronously growing cells at the start of the second division S cycle. Subsequently, at various time periods, cells were harvested, homogenized, and fractionated. The mitochondrial and the postmitochondrial supernatants were treated with ATPase antisera for the whole complex, as described under Materials and Methods. The radioactivity in the immunoprecipitates thus obtained were determined. The results are given in Figure 4B. The results are almost identical with those of the previous experiment. Thus there is evidence for the migration of the enzyme from the soluble fraction to the membrane during the late-S and G_2 phases.

There is one difference in the labeling patterns in the two experiments. When the labeled precursor is present throughout, the total radioactivity (mitochondria + PMS fractions) remains the same through the G_2 phase, whereas in pulse-labeling experiments, there is a significant drop during the late-G_2 phase. At the moment, we offer no explanations except to suggest that this may be related to the breakdown of mitochondria taking place during that period.

Artifactual Release of ATPase from the Membrane. Schatz (1968) and Tzagaloff (1969) have demonstrated accumulation of cytoplasmically made F_1 in the cytosol, in petite mutants, and in chloramphenicol-treated cells, respectively, and concluded that this accumulation was due to the release of unassembled F_1 from the mitochondria during the isolation.

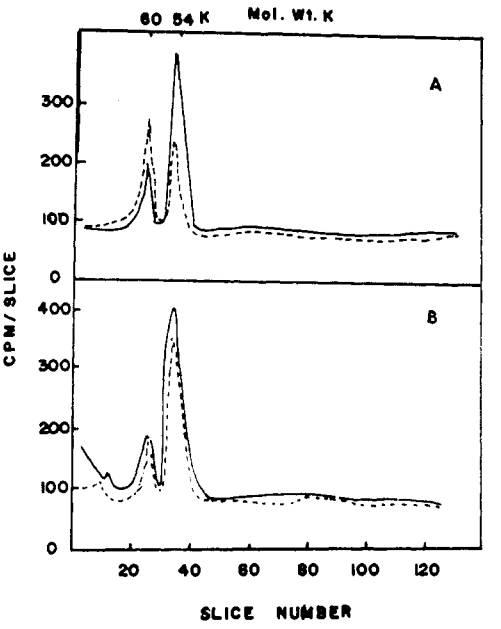

FIGURE 5: Radioactive profile of immunoprecipitates of α subunit of ATPase. The cells were labeled with ^{14}C and then followed by [^{3}H]leucine. Experimental details are given in the text. Panels A and B represent with and without EDTA treatment, respectively. (—) ^{14}C labeling. (---) ^3H labeling.

In the context of our experiments, this raises the question of whether the increase in F_1 activity in the cytosol until the mid-S phase was due to release from mitochondria or whether the cytoplasmic subunits assemble as a complex in the cytoplasm itself and are then transported.

The following experiment was designed to distinguish between these two possibilities. Cells were exposed to [^{14}C]-leucine (5 μCi/mL) for 15 min from the start of the G_1 phase. They were then transferred to a medium containing [^{3}H]-leucine (5 μCi/mL) for 5 min and chased in cold medium for another 5 min. After these operations, the cells were harvested and divided into two batches. Fractionation of one batch of cells was carried out in the normal way, but the other batch was fractionated in the same medium but containing 10 mM EDTA. The purpose of adding EDTA was to inhibit the action of a metalloprotease which is localized in the mitochondrial matrix and solubilized during grinding of the cells (G. Schatz, personal communication). This protease appears to act on mitochondrial proteins of cytosolic origin. The logic of this experiment was that in EDTA-treated cells, the cytosolically made precursors would accumulate (but not be processed). Any processed product found in the cytosol after EDTA treatment would represent the released products from the membrane.

In the above experiment described above, postmitochondrial supernatant was treated with the antiserum specific for the α subunit of F_1-ATPase. The immunoprecipitates obtained were analyzed in 10% $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 5).

Two radioactive peaks corresponding to proteins of M_r 60 $\times 10^3$ and 54 $\times$ 54 are clearly seen. The major peak at M_r 54 $\times 10^3$ corresponds to the α subunit of ATPase, and the peak at M_r 60 $\times 10^3$ represents its precursor (Maccecchini et al., 1979). When the ^{14}C, profile is looked at, there is no difference between the EDTA-treated and non-EDTA-treated cells. The extent of radioactivity present at the M_r 54 $\times 10^3$ position would therefore represent the activity released from the membrane. The amount of M_r 60 $\times 10^3$ peaks would represent the precursor still waiting to be processed, the time element

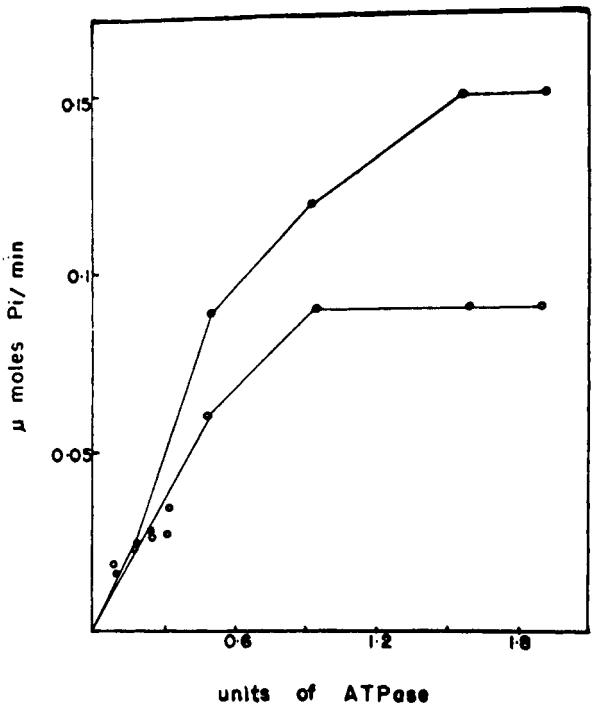

FIGURE 6: Binding of ATPase under in vitro conditions. Increasing amounts of postmitochondrial supernatant (given as units of ATPase and μmol of P_i min^{-1}) was added to equal amounts of mitochondria isolated from 2.25- (G_1) and 3.25-h (G_2) phase cells. Samples were incubated at 28 °C for 10 min and then centrifuged at 12000g for 10 min. Total activity in the resulting pellet was determined. (●) Activity in the 3.25-h (G_2) mitochondria; (○) activity in the 2.25-h (G_1) mitochondria.

of the experiment probably not being sufficient.

The ^3H profile shows a different pattern. In the absence of EDTA, there is an increased amount of M_r 54 × 10^3 peak representing the released enzyme, but about one-third of the counts is present in the M_r 60 × 10^3 peak also. In the presence of EDTA, however, the counts in the M_r 60 × 10^3 peak are slightly more than those in the M_r 54 × 10^3 peak.

These experiments suggest that (a) there is a M_r 60 × 10^3 precursor for the M_r 54 × 10^3 α subunit of ATPase which can accumulate in the cytoplasm before being transported to mitochondria and (b) there is release of at least the α subunit of F_1-ATPase, artifactually, and this should be taken into account for any interpretation.

Appearance of Cytosolic Components in Mitochondria. These experiments clearly indicate that up to the mid-S phase the cytosolically synthesized F_1-ATPase is present in the cytosol and they are detectable in the mitochondria only after the mid-S phase. At least in the case of α subunit, what is present in the cytosol is a mixture of both newly synthesized and artifactually released component. But beyond the mid-S phase, the activities become membrane bound. We addressed ourselves to the question of the parameters that govern this transfer.

(*a*) *In Vitro Studies.* The cytosolic fraction of the mid-S phase cells was prepared as the source of ATPase in the soluble form. Mitochondrial preparations were also obtained from G_1 and G_2 phase cells. These preparations were mixed with the mid-S phase cytosol in increasing concentrations and incubated at 28 °C. At the end of 10 min, the mixture was centrifuged at 15000g for 10 min to pellet down the membrane. The ATPase activities of the pellet were assayed, and the results are shown in Figure 6.

The results show that the soluble form of ATPase binds to the membrane under in vitro conditions and that the G_2 phase

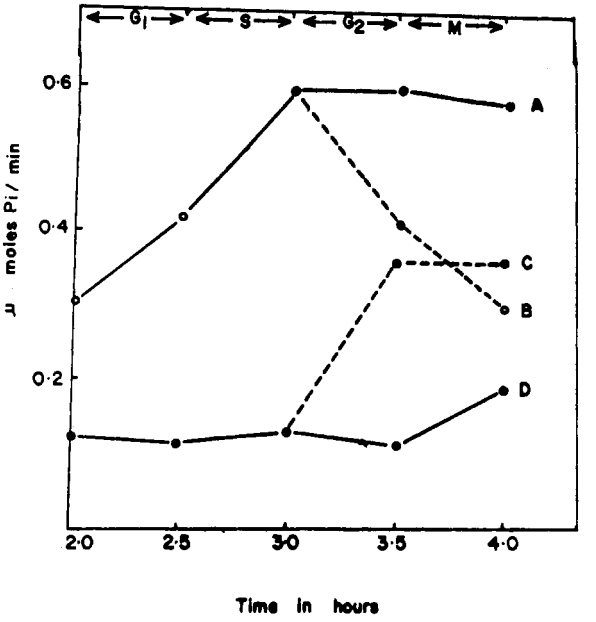

FIGURE 7: Effect of addition of Cap on the distribution of ATPase activity in mitochondrial and postribosomal supernatant fractions (PRS).

mitochondrial membranes bind almost twice (1.8-fold) the amount compared to the G_1 phase mitochondria. Thus, even if the binding is artifactual, there is a clear-cut difference between the membrane preparations from the G_1 and G_2 phases.

(*b*) *Involvement of Mitochondrial Protein Synthesis.* Another set of experiments was carried out to distinguish between the G_1 and G_2 phase mitochondrial membranes. The strategy in this series of experiments was the same as described for the continuous labeling experiments explained above except that protein synthesis inhibitors were used.

Sets of flasks were set up as before. Cap was added to one set of flasks at the end of the G_1 phase and to the other set at mid-G_2 phase. Appropriate control flasks without antibiotics were also set up. Cells were harvested at the indicated time intervals, and after processing, the ATPase activities of the mitochondrial and postribosomal fractions were assayed (Figure 7).

It can be clearly seen that addition of Cap at the G_1 phase (Figure 7, curve A) prevents the appearance of the enzyme activity in the membrane. On the other hand, addition of Cap at the beginning of the G_2 phase did not affect the process (Figure 7, curve B).

The inverse relationship is obtained in the case of mitochondria. As curves C and D of Figure 7 show, the increase in the mitochondrial fraction is observed only when Cap is added after the mid-S phase but not when Cap is added before.

These results indicate that mitochondrial protein synthesis is needed for the integration of the soluble ATPase enzyme complex into the membrane and that these components are synthesized only after the mid-S phase.

(*c*) *Role of Cytosolic Protein Synthesis.* The following experimental design was followed to study the involvement of cytosolic protein synthesis in the process. (a) One set of flasks was incubated with ^{14}C-labeled algal hydrolysate from the beginning of the G_1 to the mid-S phase. Cells were harvested and fractionated. The postmitochondrial supernatant was used as the source for radioactively labeled ATPase in the soluble form. (b) Another set of cells was treated with Cap (4 mg/mL) at the beginning of the S phase. Aliquots were removed at the mid-S, late-S, and mid-G_2 phases, and mito-

Table I: Integration of Soluble ATPase into Different Mitochondrial Membranes

	total immunoprecipitable counts		
phase	control	mitochondria CHI treated	mitochondria Cap treated
mid S (2.75 h)	600	560	500
late S (3.00 h)	800	850	520
mid G_2 (3.25 h)	2000	1760	600

chondria were obtained. (c) A third set of cells was treated with CHI (1000 μg/mL) as with chloramphenicol, and mitochondria were obtained at the mid-S, late-S, and mid-G_2 phases. The various mitochondrial pellets were incubated with the radioactive PMS fractions for 10 min at 28 °C, after which the mixtures were centrifuged. The total radioactivity in the pellets was determined, and the results are given in Table I. It is seen that addition of Cap inhibits the integration at all times, confirming the earlier result of the need for mitochondrial protein synthesis for the assembly. But addition of CHI has no influence on the integration. In both control and CHI-treated flasks, there is a sudden increase in the radioactivity between the late-S and mid-G_2 phases. Thus, the cytoplasmic involvement is only at the level of synthesis of the enzyme complex but not in its integration to the membrane, a result in agreement with other reports (Maccecchini et al., 1979).

Kinetics of Appearance of Cytosolically Synthesized Subunits in Mitochondria. Cells were exposed to ^{14}C-labeled algal hydrolysate (0.5 μCi/mL) during the G_1 phase. Earlier results (Figure 4) have shown that at this stage the enzyme activity is mainly in the soluble form. The cells were now transferred to cold "used medium" (see Materials and Methods). At different time intervals, cells were harvested and fractionated. Both the mitochondria and the PMS fraction were treated with ATPase (holoenzyme) antisera, and the immunoprecipitates were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. The radioactive profiles are given in Figure 8.

It can be seen that at 2.75 h (mid-S phase) the PMS fraction shows five distinct peaks with molecular weights of 56.5×10^3, 37×10^3, 31×10^3, 19×10^3, and 11.9×10^3, corresponding to the cytosolic subunits (Tzagaloff & Meagher, 1971). In these experiments, we have not made any attempts to distinguish between the cytosolic precursors which differ by a small molecular weight. For the same time period, the mitochondrial fraction shows hardly any peak. By 3.25 h, just after the S phase is over, the peak intensities in the PMS fraction are much reduced whereas they have become prominent in the mitochondrial fraction. This again is clear evidence that the migration of soluble enzyme takes place only after the mid-S phase.

Synthesis of Mitochondrial Subunits. Cells were pulse labeled with ^{14}C-labeled algal hydrolysate in the presence of CHI (100 μg/mL) during the different phases. After the incubation, cells were harvested, mitochondria isolated, and immunoprecipitates obtained. The total counts in the immunoprecipitates are given in Table II, which show that the mitochondrial subunits are actively synthesized only after the mid-S phase.

In a second series of experiments, similar pulse labeling was carried out in the presence of CHI, and the mitochondrial immunoprecipitates obtained were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 9). Four well-defined peaks at molecular weights of 29×10^3, 22×10^3,

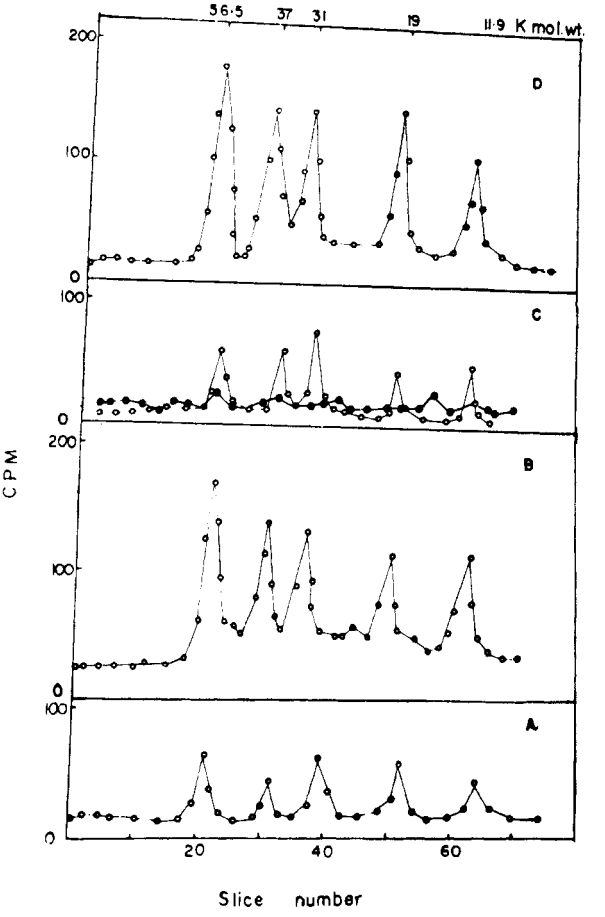

FIGURE 8: Curves A and B represent the activity in the PRS when Cap was added at 2.5 and 3.25 h, respectively. Curves C and D represent the activity in the mitochondrial fraction, after Cap was added at 3.25 and 2.5 h, respectively.

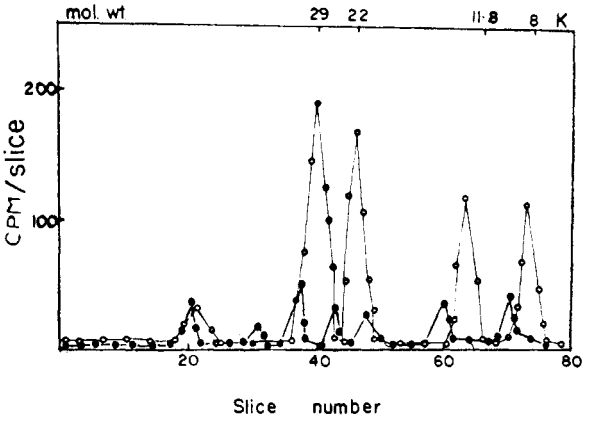

FIGURE 9: Synthesis of the mitochondrial subunits of ATPase. Cells were pulse labeled with ^{14}C-labeled chlorella hydrolysate in the presence of CHI during the late-S (2.75–3.00 h) and early-G_2 phases (3.00–3.25 h), and the immunoprecipitables from the mitochondria were analyzed on $NaDodSO_4$–polyacrylamide gels. (●) Immunoprecipitate from cells labeled during the late-S phase; (○) immunoprecipitate obtained from the cells labeled at the early-G_2 phase.

Table II: Time of Synthesis of Mitochondrial Components of ATPase

time (h)	phase	total immunoprecipitable counts
2.00–2.50	G_1	170
2.5–2.75	early S	140
2.75–3.00	late S	460
3.00–3.25	early G_2	630

11.8×10^3, and 8×10^3 were obtained at the G_2 phase, and only poor labeling is obtained at the S phase. These results are again in conformity with the above observations.

Discussion

It would be redundant to mention that the process of mitochondriogenesis involves the interaction of the cytosolic and mitochondrial protein synthesizing systems and that the products of these two systems can be independently accumulated by the use of the differentiationally acting inhibitors chloramphenicol and cycloheximide, as has been repeatedly demonstrated in several laboratories. In this laboratory, we have shown that such independently accumulated subunits of the enzymes ATPase, cytochrome oxidase, and QH_2–cyt *c* reductase can complement each other under in vitro conditions to restore the function (Chandrasekaran et al., 1980).

In this paper, we present evidence to show that during the cell cycle, the cytosolic and mitochondrial synthesis of mitochondrial protein components occur during different phases. This is established for the ATPase enzyme complex (this paper) and cytochrome oxidase (Somasundaram & Jayaraman, 1981).

Lloyd & Edwards (1977) have studied the appearance of the ATPase complex in synchronous cultures of *S. pombeii*. Their approach was to use different inhibitors like oligomycin, dicyclohexylcarbodiimide, triethyltin (which bind to the membrane factor), and Dio 9 (which inhibits the F_1-ATPase activity) and study the inhibitory pattern during various stages of the cell cycle. Since it has been shown that these inhibitors interact with different subunits of the complex, variation of the inhibitory activity would reflect on the status of the enzyme. The results obtained were interpreted by these workers either to mean conformational changes of the subunits or to reflect the time of synthesis and assembly of different subunits.

The results of our experiments demonstrate that the F_1-ATPase is synthesized during the G_1 and early-S phases but accumulates in the cytosol probably in the precursor form, as shown for the α subunit (Figure 5), for some time before being transported to mitochondria. Only after the mid-S phase are the mitochondrial counterparts synthesized, and then the cytosolic subunits are integrated to the membrane. The processing of the precursor forms is yet to be studied.

Although the immunoprecipitation and gel electrophoresis have given clear-cut evidence for the above, the conventionally accepted allotopic properties of oligomycin sensitivity and cold stability in the membrane-bound state give somewhat ambiguous results during the G_1 phase (Figure 3). Two possible reasons can be mentioned at this stage: (a) there is no quantitative correlation between oligomycin sensitivity and cold stability and (b) the allotopic behavior is not exhibited by newly synthesized products. All four mitochondrial subunits are labeled at the same time. There does not appear to be a temporal sequence, as has been shown to be the case with cyt oxidase subunits (Chandrasekaran et al., 1980; Somasundaram & Jayaraman, 1981).

Significance of Phase Difference. Barath & Kuntzel (1972) were the first to hypothesize that two phases exist in mitochondrial biogenesis, synthesis of the mitochondrial components followed by synthesis by mitochondria. The stimulatory effect of cytosolic proteins on the mitochondrial protein synthesis has been shown by several workers (Tzagaloff, 1971; Lin et al., 1974; Ibrahim & Beattie, 1976; Poyton & Kavanagh, 1976). Working with HeLa cells, Attardi and his group have shown that mitochondrial protein synthesis and mitochondrial DNA transcription occur only during the late-S and G_2 phases (Picca-Mattoccia & Attardi, 1971, 1972; England et al., 1974). The work of Lloyd & Edwards (1977) on the ATPase synthesis in *S. pombeii* has already been referred to. The results presented in this paper clearly establish this phase difference. The question therefore arises as to whether the cytoplasmically made subunits have any specific triggering effect on the synthesis of their mitochondrial counterparts. The enzyme ATPase could execute such an influence in two ways, either through satisfying the energy requirement or by direct interaction with the genetic machinery.

During respiratory adaptation, synthesis and assembly of mitochondrial components are inhibited by oligomycin (Galleotti et al., 1968). Luzikov et al. (1971) proposed that the maintenance of mitochondrial organization requires energy conservation. When ATP synthesis in the mitochondria of aerobic grown cells is inhibited and simultaneously the entry of cytosolic ATP into mitochondria is prevented by bongkrekick acid, mass formation of respiratory-deficient mutants was observed (Subik et al., 1972). Thus the continued presence of ATP inside the mitochondria seems to be a prerequisite for the protein synthesis by mitochondria. Recently it has been shown (Nelson & Schatz, 1979) that the processing of cytoplasmically made precursors is dependent on intramitochondrial ATP levels. In this context, it is interesting to note that the first event during mitochondrial assembly during derepression in yeast cells is the integration of the soluble ATPase to the membrane (Chandrasekaran et al., 1980).

On the other hand, a nuclear mutant of *S. cerevisiae* lacking F_1-ATPase has been obtained which lacks most of the mitochondrially made products. The mutation also is lethal under anaerobic conditions (Ebner & Schatz, 1973). These workers claimed that F_1 could be a nuclear organizer for triggering mitochondrial functions. A similar mutant has been isolated by Goffeau et al. (1973) in *S. pombeii*. The role of ATPase in the genetic functions of mitochondrial DNA has been extensively discussed by Mahler's group (Bastos & Mahler, 1974; Mahler & Bastos, 1974). Further studies in progress would help us localize the effect.

Transport of Products from Cytosol to Membrane. The F_1-ATPase is located on the inner side of the inner membrane, attached to the cristae. Thus the question of its transport from the cytosol to its site of function is quite intriguing. Butow and his group proposed a general model in which the mitochondrial components are inserted into mitochondria directly by ribosomes attached to the outer mitochondrial membrane, at sites where the outer and inner membranes fuse. These ribosomes specifically translate the messenger for the products needed by mitochondria but coded by the nuclear genome (Kellems & Butow, 1974; Kellems et al., 1974). But the hypothesis fails to account for accumulation of mitochondrial products in the cytoplasm (this paper; Hallermeyer et al., 1977; Harmey et al., 1977a,b). The transport of these products from the cytosol is inhibited by chlorocarbonyl cyanide phenylhydrazone. Possibilities for a steady-state pool of soluble F_1 in the cytosol has also been suggested (Tzagoloff et al., 1972). Recently Maccecchini et al. (1979) have shown that F_1 is not transported vectorially, as expected for the above model.

That the integration of the F_1-ATPase to the membrane is dependent on the presence of mitochondrially made product has been shown by several workers (Schatz, 1968; Tzagaloff, 1969; Goffeau et al., 1973). Our results show that the integration of the cytosolic subunits is affected when mitochondrial protein synthesis is affected.

Maccecchini et al. (1979) have suggested that the individual subunits of the F_1-ATPase could be synthesized first as high molecular weight precursors and then processed. In our ex-

periments using specific antisera against the α subunit of ATPase, we were able to detect the presence of a M_r 60×10^3 precursor (as against the M_r 54×10^3 product in the cytosol; see Figure 5). The above workers have also suggested that the fully assembled F_1-ATPase cannot be transported across mitochondrial membrane.

There appears, however, to be a paradox existing. Our results show an accumulation of F_1-ATPase enzyme *activity* until the mid-S phase in the cytosol and thereafter an increase in the mitochondrial membrane. This should mean either a release from the membrane or that the "precursors" themselves exhibit the enzyme activity.

The second possibility is discounted by the fact that there is so far no evidence for "precursors" showing biological activity. The ATPase enzyme is vulnerable to release from the membrane. But the data presented in Figure 5 show that both released and newly synthesized products coexist in the cytosol until the mid-S phase. Therefore the enzyme activity could be due to released products.

The overall picture seen from the results presented is that the cytosolic products accumulate in the cytosol itself until the mid-S phase, and only after this when mitochondrial subunits are made, are they integrated into the membrane. Admittedly, the present results do not throw light on the critical question of the mode and form of transport of newly synthesized cytosolic products to the mitochondrial membrane. And earlier we had demonstrated a functional complementation between independently accumulated cytosolic and mitochondrial products with reference to ATPase and cytochrome oxidase (Chandrasekaran et al., 1980). The question thus remains open.

Acknowledgments

We are grateful to Dr. G. Schatz, Biozentrum, Basel, Switzerland, for his generous gift of various antisera used.

References

Ashwell, M., & Work, T. S. (1970) *Annu. Rev. Biochem. 39*, 251.

Barath, Z., & Kuntzel, H. (1972) *Nature (London) New Biol. 240*, 195.

Bastos, R. N., & Mahler, H. R. (1974) *J. Biol. Chem. 249*, 6617.

Beattie, D. S. (1971) *Subcell. Biochem. 1*, 1.

Chandrasekaran, K., Dharmalingam, K., & Jayaraman, J. (1980) *Eur. J. Biochem. 103*, 471.

Dharmalingam, K., & Jayaraman, J. (1973) *Arch. Biochem. Biophys. 157*, 197.

Ebner, E., & Schatz, G. (1973) *J. Biol. Chem. 248*, 5379.

Elliott, S. G., & McLaughlin, C. S. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4384.

England, J. M., Pica-Mattoccia, L., & Attardi, G. (1974) in *Cell Cycle Controls* (Padilla, G. M., Cameron, I. L., & Zimmerman, A., Eds.) p 101, Academic Press, New York.

Fiske, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375.

Freedman, J. A., & Chan, S. H. P. (1978) *Mol. Cell. Biochem. 19*, 135.

Galleotti, T., Kovac, L., & Hess, B. (1968) *Nature (London) 218*, 194.

Goffeau, A., Landry, Y., Foury, F., & Briquet, M. (1973) *J. Biol. Chem. 248*, 7097.

Hallermayer, G., Zimmerman, R., & Neupert, W. (1977) *Eur. J. Biochem. 81*, 523.

Harmey, M. A., Hallermayer, G., Zimmerman, R., & Neupert, W. (1977a) *Eur. J. Biochem. 81*, 523.

Harmey, M. A., Hallermayer, G., Kore, H., & Neupert, W. (1977b) Eur. J. Biochem. *81*, 533.

Hartwell, L. H. (1974) *Bacteriol. Rev. 38* (2), 164.

Ibrahim, N. G., & Beattie, D. S. (1976) *J. Biol. Chem. 251*, 108.

Jayaraman, J., Cotman, C., Mahler, H. R., & Sharp, C. W. (1966) *Arch. Biochem. Biophys. 116*, 224.

Jayaraman, J., Dharmalingam, K., & Somasundaram, T. (1975) in *Regulation and Growth of Differential Functions in Eukaryote Cells* (Talwar, G. P., Ed.) p 3, Raven Press, New York.

Kellems, R. E., & Butow, R. A. (1974) *J. Biol. Chem. 249*, 3304.

Kellems, R. E., Allison, V. F., & Butow, R. A. (1974) *J. Biol. Chem. 249*, 3297.

Lessler, M. A. (1969) *Methods Biochem. Anal. 17*, 19.

Lin, L. H., Kin, I. C., & Beattie, D. S. (1974) *Arch. Biochem. Biophys. 160*, 458.

Linnane, A. W., Haslam, J. M., Lukins, H. B., & Nagley, P. (1972) *Annu. Rev. Microbiol. 26*, 163.

Lloyd, D., & Edwards, S. W. (1977) *Biochem. J. 162*, 581.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Luzikov, V. N., Zubatov, A. S., & Rainina, E. I. (1971) *FEBS Lett. 11*, 233.

Maccecchini, M. L., Rudin, Y., Blobel, G., & Schatz, G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 343.

Mahler, H. R., & Bastos, R. N. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2241.

Mitchison, J. M. (1971) in *The Biology of Cell Cycle*, Cambridge University Press, London.

Nelson, N., & Schatz, G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4365.

Picca-Mottaccia, L., & Attardi, G. (1971) *J. Mol. Biol. 57*, 615.

Picca-Mottaccia, L., & Attardi, G. (1972) *J. Mol. Biol. 64*, 465.

Poyton, R. O., & Kavanagh, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 11.

Schatz, G. (1968) *J. Biol. Chem. 243*, 2192.

Schatz, G., & Mason, T. L. (1974) *Annu. Rev. Biochem. 43*, 51.

Smith, D., Tauro, P., Schewizer, K., & Halvorson, H. O. (1968) *Proc. Natl. Acad. Sci. U.S.A. 60*, 936.

Somasundaram, T., & Jayaraman, J. (1979) *Indian J. Biochem. Biophys. 16*, 259.

Somasundaram, T., & Jayaraman, J. (1981) *Biochem.* (preceding paper in this issue).

Subik, J., Kacarov, J., & Kovac, L. (1972) *Biochem. Biophys. Res. Commun. 49*, 192.

Tzagoloff, A. (1969) *J. Biol. Chem. 244*, 5027.

Tzagoloff, A. (1970) *J. Biol. Chem. 245*, 1545.

Tzagoloff, A. (1971) *Curr. Top. Membr. Transp. 2*, 157.

Tzagoloff, A., & Meagher, P. (1971) *J. Biol. Chem. 246*, 7328.

Tzagoloff, A., Akai, A., & Sierra, M. F. (1972) *J. Biol. Chem. 247*, 6517.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.

Williamson, D. H. (1964) in *Synchrony in Cell Division and Growth* (Zuethen, E., Eds.) p 351, Interscience, New York.

Williamson, D. H., & Scopes, A. W. (1962) *Nature (London) 193*, 256.