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Promitochondria of Anaerobically Grown Yeast. I. Isolation and Biochemical Properties*

Richard S. Criddle† and Gottfried Schatz‡

ABSTRACT: *Saccharomyces cerevisiae* cells grown under strict anaerobiosis possess mitochondria-like particles. These particles are tentatively designated as promitochondria. They lack a respiratory chain but still contain oligomycin-sensitive adenosine triphosphatase (F_1), "structural protein," and mitochondrial deoxyribonucleic acid (density 1.685 g/cc), all of which are indistinguishable from their counterparts in aerobic yeast mitochondria. Promitochondria from anaerobically grown cells of the cytoplasmic "petite" mutant exhibit oligomycin-insensitive F_1 and thus differ from the corresponding particles of the wild-type strain. Promitochondria are present in anaerobic yeast cells regardless of whether these are grown in the presence or the absence of Tween 80 and ergosterol. These findings suggest that anaerobic growth of *S. cerevisiae* leads to a dedifferentiation, rather than a complete loss, of the mitochondrial organelles.

Aerobically grown *Saccharomyces cerevisiae* cells possess numerous typical mitochondria (Agar and Douglas, 1957; Vitols *et al.*, 1961) and an active, cyanide-sensitive respiratory chain involving the cytochromes aa_3, b, c_1, and c (Ephrussi and Slonimski, 1950; Slonimski, 1953). In contrast, the anaerobically grown cells are devoid of cyanide-sensitive respiration and the classical cytochrome complement but adaptively regain these characteristics upon aeration (Ephrussi and Slonimski, 1950; Slonimski, 1953; Chin, 1950; Lindenmayer and Estabrook, 1958; Chaix, 1961; Lindenmayer and Smith, 1964).

This oxygen-induced adaptation process obviously represents a promising experimental system for studying the formation of respiratory enzymes. In addition, it may provide a means for investigating the biogenesis of the mitochondrial membranes with which these enzymes are associated. In spite of considerable effort, however, the fate of yeast mitochondria during anaerobic growth and respiratory adaptation has remained a point of continuing discussion. Some years ago, Heyman-Blanchet *et al.* (1959) reported the isolation of "mitochondria" from anaerobically grown yeast cells but presented little evidence to support this claim. On the other hand, Wallace and Linnane (1964) concluded on the basis of

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electron microscopic studies that anaerobic yeast cells were free of mitochondria and that respiratory adaptation involved *de novo* formation of these organelles from low molecular weight precursors.

Attempts to resolve these discrepancies have thus far been unsuccessful. The numerous conflicting reports suggest, however, that the cytology of the anaerobically grown yeast cells may be influenced by the composition of the growth medium (for review, see Schatz, 1968a). Thus, Morpurgo *et al.* (1964) reported that yeast cells grown anaerobically in a simple yeast extract–glucose–salt medium similar to that used by Wallace and Linnane contained no discernible mitochondrial structures. However, the cells also appeared to be considerably damaged since they no longer adapted to oxygen and exhibited a survival rate of only 50%. Healthy anaerobic cells capable of rapid respiratory adaptation were only obtained if the growth medium was supplemented with ergosterol and oleic acid (in the form of Tween 80) in order to circumvent the oxygen-dependent synthesis (Andreasen and Stier, 1953; Bloomfield and Bloch, 1960; *cf.* Frantz and Skroepfer, 1967) of these essential membrane constituents. The anaerobic cells grown under these conditions contained numerous mitochondrial profiles prior to adaptation although they still lacked an aerobic respiratory system. While these results were subsequently confirmed by Wallace *et al.* (1968), Polakis and coworkers (1964) were unable to discern mitochondrial profiles in electron micrographs of anaerobic yeast cells even if these had been grown in the presence of added Tween 80 and ergosterol.

These discrepancies may be in part due to the fact that the anaerobic yeast cultures investigated by different workers were grown under different conditions and isolated at different points in their growth cycle. Even more important, the cells were often harvested in a way that would be expected to allow for considerable respiratory adaptation. However, the most serious ambiguities appear to arise from the present limitations of electron microscopy. Positively stained mitochondrial membranes lack any morphological features that clearly differentiate them from other membrane types. As a consequence, the identification of mitochondria in positively stained and embedded specimens generally presupposes the presence of well-defined *cristae mitochondriales* which may be sparse or absent under certain physiological conditions (*cf.*, *e.g.*, Yotsuyanagi, 1962). Additional problems are introduced by the fact that anaerobic growth alters the lipid composition of the yeast cells (Kováč *et al.*, 1967; Paltauf and Schatz, 1969) and thereby may modify the staining characteristics of the intracellular membranes. It would thus seem rather risky to ascertain the absence of mitochondria by electron microscopy of stained and sectioned anaerobic yeast cells.

The unique biochemical properties of mitochondrial membranes provide an alternate approach for exploring the occurrence of these membranes in anaerobic yeast cells. In a preliminary study, Schatz (1965) separated the subcellular particles from anaerobic yeast cells into distinct populations and determined their enzymic characteristics. These experiments suggested that the anaerobic cells contained subcellular particles which exhibited many properties typical of mitochondria. It was therefore proposed that the oxygen-induced synthesis of typical mitochondria in yeast proceeded *via* differentiation of incomplete mitochondria rather than mitochondrial *de novo* formation and that it was thus similar to the better characterized light-induced formation of chloroplasts from "proplastids." Since the term "proplastid" is well established (*cf.*, *e.g.*, Goodwin, 1966), the mitochondria-like particles from anaerobic yeast cells were correspondingly termed "promitochondria." This term emphasizes the fact that the particles are closely related to the respiring mitochondria of aerobic yeast cells but at the same time lack many of their most distinguishing features.

The present study was prompted by recent advances in the characterization of the mitochondrial inner membrane (for review, *cf.* Racker, 1968; Pullman and Schatz, 1967). These advances permit an unequivocal identification of mitochondrial inner membranes in anaerobic yeast and thus furnish a promising experimental basis for consolidating and extending our earlier work.

Materials and Methods

Yeast Strains and Culture Media. Unless specified otherwise, the experiments described in this paper were carried out with the wild-type *S. cerevisiae* strain D 273–10 B (α $P\rho^+$, haploid) or the corresponding cytoplasmic "petite" mutant D 273–10 B-1 (Sherman, 1965). Both strains were routinely grown in a semisynthetic medium (Schatz and Klima, 1964) containing 10% glucose, 0.26% Tween 80, 12 ppm of ergosterol, and a few drops of a silicone antifoam emulsion. In a few specified instances, the concentration of glucose was lowered to 0.8% or the addition of Tween 80 and ergosterol was omitted. For the pulse-labeling experiments, a diploid, respiratory-competent lysine auxotroph of *S. cerevisiae* was employed. This was grown in a defined medium (Reiff *et al.*, 1960) which contained 10% glucose, 0.26% Tween 80, and 12 ppm of ergosterol.

Growth and Harvesting of Anaerobic Yeast Cells. Purified nitrogen (less than 0.01% oxygen) was freed of the last traces of oxygen by slow successive passage through an alkaline dithionite solution and a dense suspension of commercial baker's yeast cells in buffered 2% ethanol (Figure 1). The oxygen-free nitrogen then passed through the culture carboy and escaped through a mercury trap. After the culture medium (total volume 10 l.) had been inoculated with approximately 10^8 aerobic yeast cells, it was flushed with nitrogen for 8–10 hr. Stopcock I was then closed and growth of the cells followed turbidimetrically on samples collected through an outlet in carboy L (for the sake of clarity, this outlet is not included in Figure 1). The cells were grown at 28° and harvested in their early stationary phase. At this point, the cultures contained between 6×10^{11} and 1×10^{12} cells, corresponding to a 6000–10,000-fold dilution of the inoculum. (In the absence of Tween 80 and ergosterol, the yield of cells was about 10–20 times smaller.) For harvesting, the mercury trap was closed and the rapidly stirred culture carboy chilled in an eth-

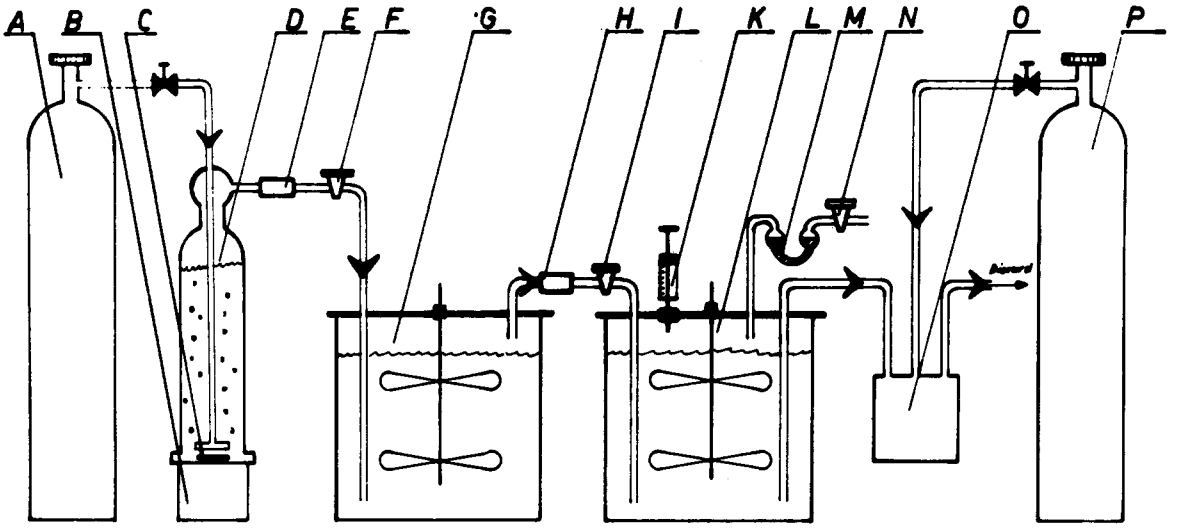

FIGURE 1: Schematic illustration of equipment used for the anaerobic growth and isolation of yeast cells. (A) Tank containing highly purified nitrogen (>99.99% N_2); (B) magnetic stirring motor; (C) magnetic stirring bar; (D) washing bottle containing 1 l. of alkaline, 10% (w/v) $Na_2S_2O_4$ solution; (E, H) cotton plugs; (F, I, N) stopcocks; (G) carboy containing 2 kg of commercially grown baker's yeast cells suspended in 10 l. of 50 mM KH_2PO_4–2% ethanol; (K) rubber seal with syringe for poisoning the anaerobic culture; (L) culture carboy containing 10 l. of culture medium (see text). The carboys G and L are part of a New Brunswick fermentor. They are stirred at 200 rpm and thermostated at 28°; (M) mercury trap; (O) continuous-flow rotor of an MSE high-speed centrifuge operating at −2 to −4° and at 18,000 rpm; and (P) tank containing liquid carbon dioxide. For the sake of clarity, the drawing does not accurately reflect the relative size of the various parts.

anol–Dry Ice mixture until the medium began to freeze (internal temperature about −5°). An oxygen-free cycloheximide solution (15 ml; 15 mg/ml) was then injected through a rubber seal. In some experiments, in which only mitochondrial ATPase or cytochrome spectra were studied, the culture received in addition 5 ml of 0.5 M iodoacetate (pH 7.4). In control experiments it was found that cells poisoned with both cycloheximide and iodoacetate incorporated [^{14}C]leucine and [^{3}H]uridine at a rate which was only 0.0001 and 5%, respectively, of that observed with nonpoisoned cells. The poisoned cells also failed to adapt to oxygen even after aeration for 8 hr. With the purified nitrogen as propellant, the chilled, poisoned cell culture was then forced through a continuous-flow rotor operating at −2 to −4° in an atmosphere of carbon dioxide. The sedimented cells were washed twice with a solution containing 0.25 M mannitol, 20 mM Tris-SO_4 (pH 7.4), 2 mM EDTA, 50 μg/ml of cycloheximide, and 0.1% bovine serum albumin. They were suspended in the above medium and immediately homogenized for 20 sec at 0° as described earlier (Schatz, 1967). Aerobically grown yeast cells were obtained as outlined in a previous communication (Schatz, 1968b).

Isolation of Mitochondria and Promitochondria. Unless stated otherwise, all manipulations were carried out at 0–4°. For the isolation of mitochondria, homogenates of the aerobically grown cells were centrifuged for 20 min at 20,000*g* in an MSE high-speed centrifuge. The sedimented "crude mitochondria" were washed once with the homogenization medium by recentrifugation and suspended in this medium to a particle concentration of approximately 40 mg/ml. Aliquots (2–5 ml) of this suspension were then layered on a linear, continuous sucrose gradient (20–70% (w/v) sucrose containing 20 mM Tris-SO_4 (pH 7.4) and 2 mM EDTA) and centrifuged for 2.5 hr at 25,000 rpm in the Spinco SW25 rotor. The mitochondria, which had equilibrated at a density of 1.16–1.18 g/cc, were collected by means of a syringe, diluted three- to sixfold with homogenization medium, and collected by centrifugation for 20 min at 105,000*g*.

For the isolation of promitochondria, homogenates of the anaerobically grown cells were centrifuged for 90 min at 105,000*g* in a Spinco no. 30 rotor. The sedimented particles were suspended in the homogenization medium and recentrifuged. The "crude particle fraction" thus obtained was suspended in homogenization medium by 50 up-and-down strokes in a tight-fitting Potter-Elvehjem homogenizer and purified by sucrose gradient centrifugation as outlined above. However, because of the smaller size of the isolated promitochondria, the duration of gradient centrifugation was extended to 12–16 hr. Moreover, the various particle populations recovered from the gradient were collected by centrifuging for 60 min at 105,000*g*. In a few specified experiments the promitochondria were flotated in a linear "Urografin" gradient (1.10–1.20 g/cc; Schatz *et al.*, 1964; Schatz, 1965).

Biochemical Preparations. An antiserum against purified mitochondrial ATPase (F_1) from aerobic yeast mitochondria was prepared as described earlier (Schatz *et al.*, 1967). "Structural protein" was isolated from mitochondia and promitochondria according to Criddle *et al.* (1962). The antiserum against structural protein from respiring yeast mitochondria was a generous gift of Drs. H. Tuppy and P. Swetly. ^{32}P-Labeled yeast ribosomes were donated to us by Dr. E. Wintersberger.

Isolation and Characterization of (Pro)mitochondrial DNA. DNA was isolated from mitochondria and pro-

mitochondria according to Moustacchi and Williamson (1966). The mitochondria were purified by sedimentation in a sucrose gradient, the promitochondria by flotation in a "Urografin" gradient. The DNA was characterized by centrifugation in a CsCl gradient in a Spinco Model E analytical ultracentrifuge (Vinograd and Hearst, 1962). Buoyant densities were calculated using the position of *Pseudomonas aeruginosa* DNA (density 1.727 g/cc) as a reference (Schildkraut *et al.*, 1962). Densitometric tracings of the ultraviolet absorption photographs were employed to estimate boundary positions and relative concentrations.

Enzyme Assays. The following enzymic activities were measured according to published procedures (assay temperature in parentheses): ATPase (30°; Pullman *et al.*, 1960), succinate and NADH oxidase (22–24°; Schatz, 1968b), cytochrome *c* oxidase (22–24°; Birkmeyer, 1968), succinate- and NADH-ferricyanide reductase (30°; Schatz, 1967; De Bernard, 1957), and succinate–cytochrome *c* reductase (30°; Schatz and Klima, 1964). Succinate dehydrogenase was assayed by a modification of the procedure of Nachlas *et al.* (1960). The assay medium contained 0.1 M sodium potassium phosphate buffer (pH 7.4), 0.1% Triton X 100, 20 mM potassium succinate, 0.4 mM 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride, and 0.04–0.4 mg/ml of phenazine methosulfate. The medium of the reference cuvet was identical except that succinate was replaced by an equimolar amount of potassium malonate. The final volume was 3.0 ml, the temperature 30°. After adding identical amounts of enzyme to both assay and reference cuvet, the succinate-dependent reduction of the tetrazolium salt was followed spectrophotometrically at 540 mμ. Activity was extrapolated to infinite concentration of phenazine methosulfate. Respiration of yeast cells was measured polarographically at 22–24° in 0.1 M Tris-SO_4 (pH 7.4)–50 mM glucose.

Incorporation of Radioactive Precursors. The incorporation of [^{14}C]leucine and [^{3}H]uridine into whole yeast cells was measured essentially as described by Fukuhara (1967b). The labeling of individual subcellular fractions by [^{14}C]lysine was determined as follows. A magnetically stirred suspension of yeast cells (50 mg wet weight/ml in 50 mM potassium phthalate buffer (pH 5.5)–50 mM glucose–1 M ethanol, and inhibitors as indicated), was incubated anaerobically at room temperature for 45 min. The suspension was then vigorously aerated and simultaneously mixed with L-[1-^{14}C]lysine (35.4 mCi/mole; final concentration 1 μCi/ml). (In the anaerobic labeling experiments, aeration was omitted.) After 1 min, the cell suspension was diluted 50 times with an ice-cold solution containing 0.25 M mannitol–20 mM Tris-SO_4 (pH 7.4)–2 mM EDTA–1 mM iodoacetate–10 mM nonlabeled L-lysine and 0.1% bovine serum albumin. The cells were collected by centrifugation, washed once in the above medium, and homogenized as described in an earlier section. To the isolated subcellular fractions, bovine serum albumin (final concentration 2 mg/ml) was added as a carrier and the proteins were precipitated with 5% trichloroacetic acid. They were washed twice with 5% trichloroacetic acid by suspending and centrifuging, and dissolved in 2 ml of 1 M NaOH containing 1 mM nonlabeled L-lysine. After neutralization with 5 N H_2SO_4, the proteins were precipitated with an equal volume of 10% trichloroacetic acid. The solution and the precipitate were heated to 85° for 15 min and again centrifuged. The precipitate was washed twice with ethanol–ether (3:1), dried in a vacuum desiccator, and dissolved in 0.4 ml of concentrated formic acid. Aliquots (0.1 ml) were counted in 10 ml of dioxane scintillation fluid (Bray, 1960) using a Packard liquid scintillation counter.

Disc Gel Electrophoresis of Structural Protein. Structural protein was reduced with 1 mM dithioerythritol in the presence of 8 M urea and then treated with 50 mM iodoacetate for 1 hr at pH 8.5. The resultant soluble protein preparation was dialyzed and subjected to electrophoresis in an 8% polyacrylamide gel according to a modified Ornstein–Davis procedure (Ornstein, 1964; Davis, 1964). The stacking buffer was 0.1 M potassium phosphate buffer (pH 7.3), the running buffer 0.1 M potassium phosphate buffer (pH 8.9) containing 0.03% sodium dodecyl sulfate. The current was maintained at 1 mA during stacking and at 3 mA during running. The bands were stained for 1 hr with 0.5% buffalo blue black in 50% ethanol–15% acetic acid–35% water. Excess dye was removed by washing the gels first with 10% acetic acid and then with 25% ethanol–7% acetic acid.

Miscellaneous Determinations. Protein was measured according to Lowry *et al.* (1951) in the presence of 0.33% sodium deoxycholate to solubilize particulate protein. RNA was assayed by the orcinol method (Mejbaum, 1939).

Results

Properties of the Anaerobically Grown Yeast Cells. Experiments with anaerobic yeast cells require the following minimal precautions: (1) maintenance of strict anaerobiosis during cell growth; (2) a sufficiently small inoculum in order to ensure satisfactory dilution of the added aerobic cells during anaerobic growth; and (3) prevention of respiratory adaptation during harvesting of the cells. These requirements have generally not been met in previous studies of anaerobic yeast. It would appear, however, that the yeast cells isolated by the present procedure were truly anaerobic and could not undergo significant respiratory adaptation prior to homogenization.

When tested polarographically, the anaerobically grown cells respired glucose at 5–20% of the rate observed with aerobically grown cells. However, respiration of the anaerobic cells was insensitive to 1 mM KCN and decreased progressively as the oxygen concentration of the medium fell below 0.3 μatom/ml. Since respiration of the aerobic cells is over 95% inhibited by 1 mM KCN and linear down to the lowest measurable oxygen concentrations, the oxidase operative in the anaerobic cells is clearly different from the cytochrome oxidase of the aerobic ones (*cf.* also Lindenmayer and Smith, 1964).

This conclusion is supported by low-temperature spectra of the dithionite-reduced yeast cells (Figure 2). As noted previously by several other authors (*cf.* Slon-

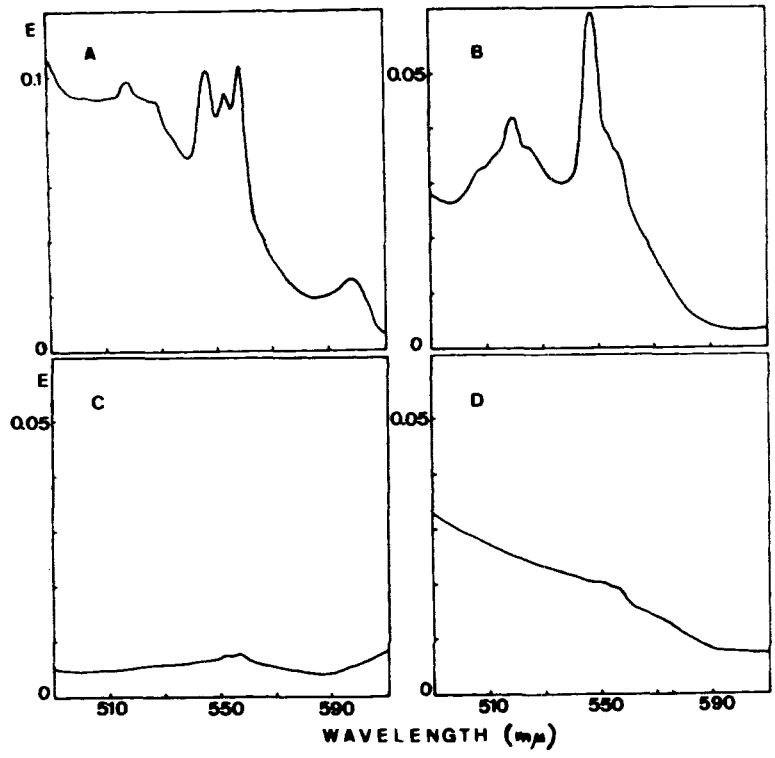

FIGURE 2: Absolute low-temperature spectra of *S. cerevisiae* cells. (A) Wild-type grown aerobically; (B) "petite" mutant grown aerobically; (C) wild-type grown anaerobically; and (D) "petite" mutant grown anaerobically. All cells had been grown to the stationary phase in the presence of 10% glucose as described under Materials and Methods. The packed yeast cells were then evenly suspended in a medium containing 0.25 M mannitol, 20 mM Tris-SO_4 (pH 7.4), 2 mM EDTA, and 50 μg/ml of cycloheximide. Each milliliter of the final suspension contained 500 mg (wet weight) of cells. Aliquots (0.2 ml) of this suspension were diluted with 0.3 ml of 0.1 M phosphate buffer (pH 7.4), reduced with a few grains of sodium dithionite, and diluted further with 0.5 ml of glycerol. The absolute absorption spectra of the cell suspensions were then recorded at the temperature of liquid nitrogen and at a light path of 1 mm essentially as described by Estabrook (1961).

TABLE I: Properties of ATPase Associated with *S. cerevisiae* Promitochondria.[a]

Source of Promitochondria	ATPase Act. (μmoles of ATP/min per mg of protein)			
	No Addition	+ F_1 Inhibitor	+ F_1 Antiserum	+ Control Serum
Wild-type grown with lipid supplement	1.35	0.12	0.21	1.40
Wild-type grown without lipid supplement	0.71	0.069	0.11	0.68
"Petite" mutant grown with lipid supplement	0.52	0.078	0.041	0.55

[a] The particles were isolated as described under Materials and Methods except that all manipulations subsequent to cell homogenization were carried out at room temperature. The amounts of inhibitors added were as follows: F_1 inhibitor, 0.26 mg; F_1 antiserum (or controlserum), 4.3 mg. The effect of inhibitors on mitochondrial ATPase was determined as described earlier (Schatz, 1968b) except that the particles were sonicated for 10 sec prior to being mixed with inhibitor.

imski, 1953, for review of the early work), the anaerobic cells lack the cytochromes aa_3, *b*, *c*, and c_1 and contain only small amounts of a pigment with absorption bands at 551.5 and 556 mμ. This pigment is undoubtedly identical with the "cytochrome b_1" described by others (Slonimski, 1953; Chaix and Heyman-Blanchet, 1957; Lindenmayer and Estabrook, 1961). Its possible function will be discussed in a later section. Under the present experimental conditions, the anaerobic yeast cells thus exhibit a remarkably simple absorption spectrum and contain at least twenty times less spectroscopically detectable hemoproteins than the aerobic cells.

Isolation of Promitochondria from the Anaerobic Cells. Upon centrifugation of a homogenate of anaerobically

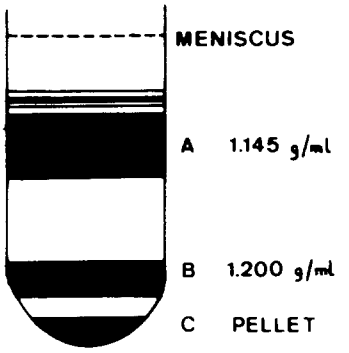

FIGURE 3: Purification of promitochondria from wild-type yeast in a sucrose density gradient.

grown yeast cells for 90 min at 105,000*g*, 26–30% of the homogenate protein was recovered in the particulate pellet. Flotation of these "crude particles" in a "Urografin" gradient yielded two major bands and a tightly packed, copious pellet. The most prominent band has earlier been shown (Schatz, 1965) to consist of promitochondria. It was found at a density of 1.15 g/cc, identical with that of respiring yeast mitochondria in this type of gradient (Schatz *et al.*, 1964).

Sedimentation of the "crude particles" in a sucrose gradient produced a closely similar result (Figure 3). Again, the promitochondria accounted for the most prominent band and equilibrated at a density of 1.145 g/cc. The purified promitochondria accounted for 14–20% of the protein present in the "crude particle" fraction. As reported earlier (Schatz, 1965), the sedimentation *velocity* of promitochondria in a sucrose gradient was considerably lower than that of normal aerobic yeast mitochondria.

Identification of Mitochondrial ATPase (F_1) in the Promitochondria. Promitochondria purified by sucrose gradient centrifugation exhibit ATPase activity (Table I). This ATPase could be unequivocally identified as mitochondrial ATPase (F_1) since it was almost completely inhibited by the naturally occurring F_1 inhibitor of Pullman and Monroy (1963) as well as by a specific antiserum (Schatz *et al.*, 1967) against purified F_1 from aerobic yeast mitochondria (Table I). Moreover, the ATPase associated with promitochondria of the wild type is cold stable and inhibited by concentrations of oligomycin similar to those blocking the ATPase of aerobic yeast mitochondria (Figure 4). Up to 90% of the F_1-ATPase present in a homogenate of wild-type anaerobic yeast was recovered in the purified promitochondria. These results were obtained regardless of whether the yeast cells had been grown in the presence or the absence of Tween 80 and ergosterol. Similar experiments with anaerobically grown cells of a cytoplasmic "petite" mutant showed the promitochondria of these cells to contain oligomycin-insensitive and cold-labile F_1. These particles thus exhibit the characteristic inner membrane lesion which had previously been detected (Schatz, 1968b; Kováč and Weissová, 1968) with mitochondria of the aerobic "petite" cells (Table I, Figure 4).

In order to assess the *total* F_1 content of wild-type yeast cells grown under different conditions, the cell

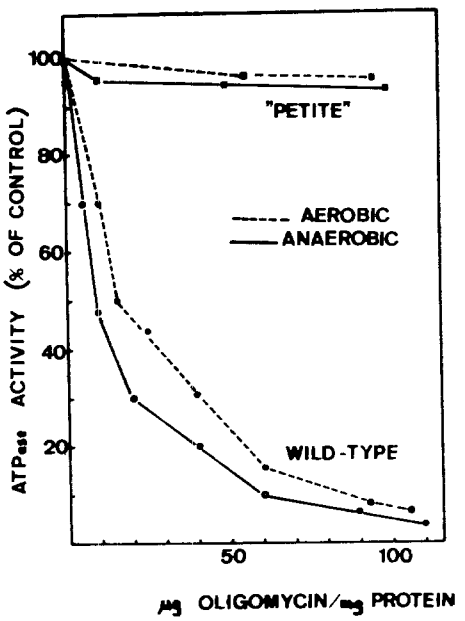

FIGURE 4: Effect of oligomycin on the ATPase of mitochondria and promitochondria from wild-type and "petite" mutant *S. cerevisiae*. Mitochondria and promitochondria were isolated as described under Materials and Methods except that all steps subsequent to the homogenization of the cells were carried out at room temperature. Oligomycin sensitivity of mitochondrial ATPase was measured as described earlier (Schatz, 1968b).

homogenates were freed from inorganic phosphate by passage through short columns of Sephadex G-25 and assayed for ATPase activity sensitive to the F_1 antiserum. Under our experimental conditions, homogenates of the aerobic cells (grown in the absence or presence of Tween 80 and ergosterol) contained 20–35 units (micromoles of ATP cleaved per minute) of F_1-ATPase/100 mg of protein. The corresponding values for homogenates of anaerobic cells grown in the presence and the absence of a lipid supplement were 7–18 and 2–6, respectively. In all cases, the F_1-ATPase of the wild-type homogenates was fully cold stable and oligomycin sensitive. While the presence of unknown amounts of endogenous F_1 inhibitors in the homogenates precludes a definite interpretation of these results, it is nevertheless clear that anaerobic yeast cells invariably contain significant quantities of membrane-bound F_1.

The subcellular particles equilibrating at a density of 1.20 g/cc (Figure 3) also exhibit ATPase activity. However, this ATPase is insensitive to the F_1 antiserum and the F_1 inhibitor of Pullman and Monroy (1963) and thus clearly different from mitochondrial ATPase. The particles appear to be identical with the nonmitochondrial membranes which are also present in aerobic yeast cells and have been described in previous reports (Schatz *et al.*, 1963; Schatz, 1968b).

Other Enzymic Activities Associated with Promitochondria. In addition to F_1, the promitochondria from wild-type yeast also contain ferrochelatase (*cf.* Schatz, 1965) as well as NADH–ferricyanide reductase and succinate dehydrogenase (Table II). The occurrence of succinate dehydrogenase was studied in some detail as the pres-

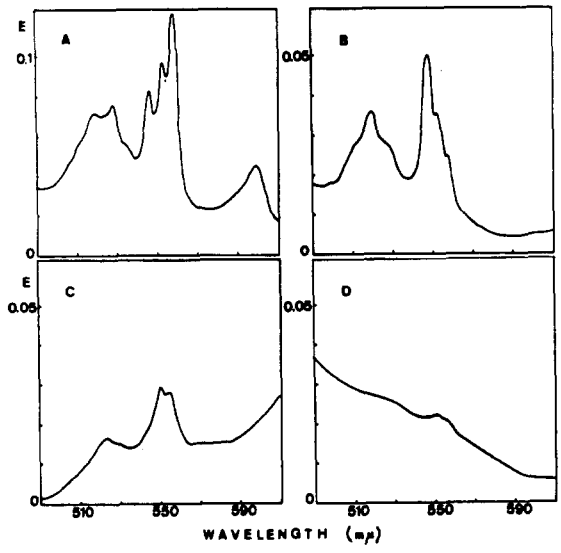

FIGURE 5: Absolute low-temperature absorption spectra of mitochondria and promitochondria. (A) Mitochondria of aerobic wild type; (B) mitochondria of aerobic "petite" mutant; (C) promitochondria of anaerobic wild-type; and (D) promitochondria of anaerobic "petite" mutant. Aliquots (0.2 ml) of the particle suspensions in 0.25 M mannitol–20 mM Tris-SO_4 (pH 7.4)–2 mM EDTA (A, 13.5 mg of protein/ml; B, 24.0 mg/ml; C, 27.3 mg/ml; and D, 35.5 mg/ml) were diluted further with 0.5 ml of glycerol. The absorption spectra were then recorded exactly as described in Figure 2.

TABLE II: Enzyme Content of Mitochondria and Promitochondria from Wild-Type *S. cerevisiae*.[a]

Enzyme Activity	Specific Act. (μmoles of substrate transformed/min/mg of protein)	
	Mitochondria	Promitochondria
F_1-ATPase	3.94	0.95
Succinate dehydrogenase	0.138	0.063
NADH–ferricyanide reductase	0.93	0.293
Succinate–ferricyanide reductase	0.144	0.0061
+6.5 μg of antimycin A	0.022	0.0060
Succinate–cytochrome *c* reductase	0.068	0.000
Succinate oxidase	0.100	0.000
NADH oxidase	0.75	0.002
+1 mM KCN	0.003	0.003
Cytochrome *c* oxidase	1.10	0.002

[a] The experimental conditions were as described under Materials and Methods except that Tween 80 and ergosterol were also present during aerobic growth of the cells.

ence of this enzyme in anaerobic yeast has aroused considerable discussion (Slonimski, 1953; Hebb *et al.*, 1959; Schatz, 1965; Lukins *et al.*, 1966; Hauber and Singer, 1967). Homogenates of anaerobic yeast cells grown in the presence of Tween 80 and ergosterol exhibited approximately 50% of the succinate dehydrogenase activity of the corresponding aerobic homogenates. About two-thirds of this activity was recovered in the "crude particle" fraction and, upon sucrose gradient centrifugation, was exclusively associated with the promitochondrial band. The remaining one-third of the homogenate activity was not sedimented by 90-min centrifugation at 105,000*g* even though this centrifugal force sedimented over 95% of the F_1–ATPase present in the homogenates.

The succinate dehydrogenase activity of the purified promitochondria was completely inhibited by malonate (K_i in the order of 10^{-7} M). The apparent K_m for succinate was approximately 10^{-4} M.

These results are in contrast to those of Lukins *et al.* (1966) who claimed that anaerobic yeast cells grown with glucose as the main carbon source are invariably devoid of succinate dehydrogenase. On the other hand, if our yeast cells were grown anaerobically in the absence of a lipid supplement, their succinate dehydrogenase content was indeed very low. On a protein basis, the isolated promitochondria from these cells contained 10–15 times less succinate dehydrogenase activity than the promitochondria from cells grown in a lipid-enriched medium. The concentration of succinate dehydrogenase in anaerobic yeast cells is thus profoundly affected by the lipid composition of the growth medium.

Regardless of the composition of the culture medium, the promitochondria lacked an integrated respiratory chain. Thus, they were consistently incapable of oxidizing succinate with either cytochrome *c* or molecular oxygen as acceptor. They also exhibited an extremely low activity of succinate–ferricyanide reductase (Table II). In mitochondria from aerobically grown wild-type cells, this activity is almost as high as that of succinate dehydrogenase itself and is 75–85% inhibited by antimycin A (*cf.* also Schatz and Racker, 1966). With aerobic yeast mitochondria, ferricyanide is thus reduced by succinate preferentially at the antimycin-sensitive factor or at its oxygen side. The lack of antimycin-sensitive succinate ferricyanide reductase and succinate oxidase in the promitochondria therefore indicates that the succinate dehydrogenase of these particles is not linked to a functional respiratory chain. This conclusion is also in line with the fact that promitochondria are devoid of cytochrome *c* and NADH oxidase (Table II) as well as of the cytochromes aa_3, *b*, c_1, and *c* (*cf.* below).

Redox Pigments Associated with Promitochondria. The low-temperature absorption spectra of the isolated promitochondria were qualitatively indistinguishable from those of the corresponding intact cells (*cf.* Figure 5, which also includes the spectra of the corresponding aerobic mitochondria). However, the concentration of "cytochrome b_1" was considerably higher in the promitochondria than in the whole cells. "Cytochrome b_1" appeared to be tightly membrane bound since prolonged sonication of the isolated promitochondria failed to solubilize the pigment. Under our experimental conditions, dithionite was by far the best reductant for membrane-

TABLE III: Flavin Content of Promitochondria from Wild-Type *S. cerevisiae*.[a]

Reducing Agent	mμmoles of Flavin Reduced/mg of Protein
Succinate	0.019
NADH	0.155
$Na_2S_2O_4$	0.170
Total	0.344

[a] The flavin content was determined by dual-wavelength spectroscopy (Chance and Williams, 1956) at a particle concentration of 2.95 mg/ml.

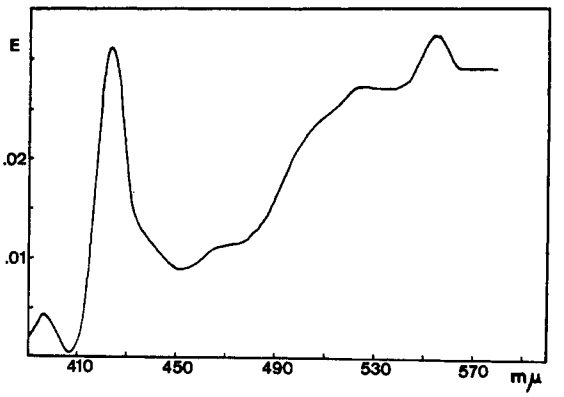

FIGURE 6: Reduced minus oxidized difference spectrum of promitochondria from wild-type yeast. The test sample received 0.2 ml of wild-type promitochondria (23.6 mg/ml) in 0.25 M mannitol–20 mM Tris-SO_4 (pH 7.4)–2 mM EDTA, 0.3 ml of 0.1 M phosphate buffer (pH 7.4), a few grains of solid sodium dithionite, and 0.5 ml of glycerol. The reference sample received all of the above components except the sodium dithionite. The difference spectrum was recorded at the temperature of liquid nitrogen (Estabrook, 1961).

bound cytochrome b_1. NADH reduced it only poorly whereas succinate was completely ineffective (*cf.* also Heyman-Blanchet, 1963).

As would be expected from the content of succinate– and NADH–dehydrogenase, the isolated promitochondria contain appreciable amounts of spectroscopically detectable flavin (Figure 6). A preliminary quantitative estimation of the various classes of flavoproteins is given in Table III. It should be pointed out that the values of this table are still subject to some uncertainty as spectroscopic flavin measurements on particulate preparations are not always reliable. It seems justified to conclude, however, that promitochondria do contain flavin in amounts not greatly different from those present in aerobic yeast mitochondria (*cf.*, *e.g.*, Mackler, 1967).

Identification of Mitochondrial "Structural Protein" in Promitochondria. A mitochondrial fraction termed structural protein accounts for approximately one-third of the total mitochondrial protein and appears to be another characteristic component of mitochondrial membranes (Criddle *et al.*, 1962). It was found that an antiserum against "structural protein" from aerobic yeast mitochondria (Tuppy *et al.*, 1968) agglutinated purified promitochondria (Table IV). No comparable agglutination was observed if the antiserum was replaced by a corresponding nonimmune serum or if it was tested against isolated nonmitochondrial membranes from the anaerobic cells (band B of Figure 3; *cf.* Schatz *et al.*, 1963; Schatz, 1968b). The antiserum also specifically agglutinated human erythrocytes coated with "structural protein" from wild-type promitochondria (Table IV).

When the "structural protein" fractions (Criddle *et al.*, 1962) from respiring yeast mitochondria and from promitochondria were compared by polyacrylamide gel electrophoresis, the patterns illustrated in Figure 7 were obtained. Both preparations exhibited a major band, some protein moving near the dye front, as well as three very faint bands of low mobility. We consider it immaterial whether these different bands represent impurities (*cf.*, *e.g.*, Haldar *et al.*, 1966; Tuppy *et al.*, 1968) or merely different aggregates of "structural protein" itself. In the present context we interpret the electrophoretic and immunological data from a purely operational point of view and wish to emphasize that the "structural protein" fractions from mitochondria and promitochondria appear to be closely similar, if not identical. It should also be mentioned that the amounts of "structural protein" recovered from mitochondria and promitochondria were essentially the same (0.35–0.45 mg/mg of particle protein).

Identification of Mitochondrial DNA in the Promitochondria. As noted earlier (Schatz, 1965), promitochondria purified by flotation in a "Urografin" density gradient contain small amounts of DNA. Upon centrifugation in a CsCl gradient, a large proportion of this DNA equilibrates at a density of 1.685 g/cc and thus represents mitochondrial DNA (Figure 8). Some contaminating nuclear DNA with a density of 1.700 g/cc is also present. A quantitative evaluation of the densitometric traces indicates that the amount of mitochondrial DNA associated with the isolated promitochondria of strain D 273-10 B is approximately 5 μg/mg of particle protein. This value is quite similar to that determined for the corresponding aerobic mitochondria (approximately 4 μg/mg of protein; Viehauser *et al.*, 1965). Since mitochondrial DNA is one of the most intrinsic and distinguishing components of the mitochondrial organelle, the present result furnishes a particularly persuasive argument for the notion that the promitochondria described here are closely related to the respiring mitochondria of aerobic yeast cells.

Amount of Promitochondria in the Anaerobic Yeast Cells. The data of Table V indicate that the amount of promitochondria within the anaerobic cells is similar to the amount of mitochondria in the same cells grown aerobically. This result excludes the possibility that the promitochondria merely represent degenerated remnants of the mitochondria added with the aerobic cells of the inoculum. Moreover, the increased level of promitochondria in the anaerobic cells grown in a lowered concentration of glucose (0.8%) constitutes direct evidence that glucose represses the synthesis of mitochon-

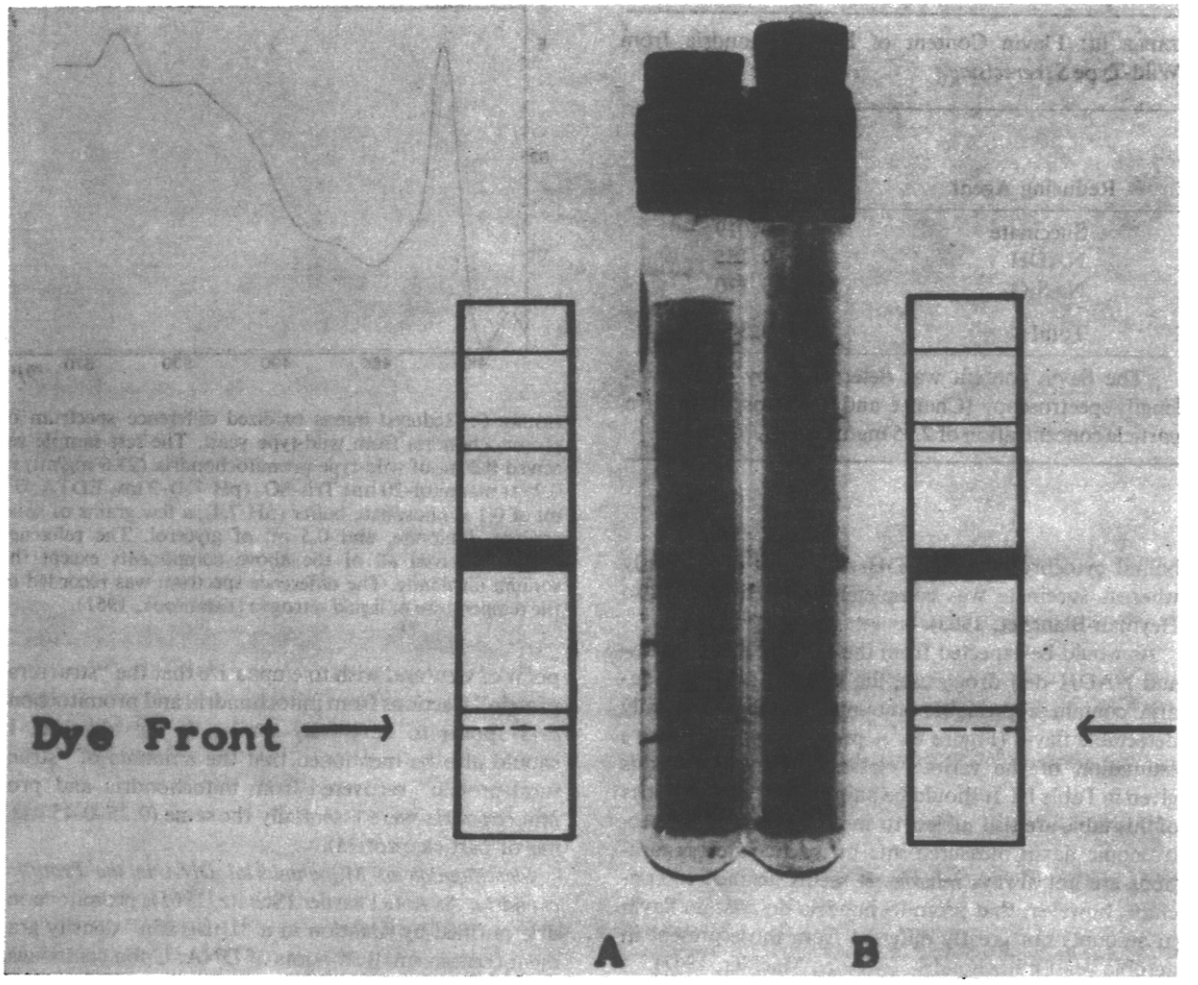

FIGURE 7: Disc gel electrophoresis patterns of "structural proteins" isolated from mitochondria (A) and promitochondria (B). See Materials and Methods for details.

TABLE IV: Immunological Demonstration of Mitochondrial "Structural Protein" in *S. cerevisiae* Promitochondria.

Antigen Used in Agglutination Experiment	Serum	Agglutination at Dilution of Serum							
		1	2	4	8	16	32	64	128
A. Promitochondria[a]	Antiserum	+	+	+	+	+	+	±	−
	Control serum	+	−	−	−	−	−	−	−
B. "Structural protein" from promitochondria[b]	Antiserum	+	+	+	+	+	+	+	
	Control serum	−	−	−	−	−	−	−	

[a] In expt A, 0.05 ml of a suspension of wild-type promitochondria (2 mg/ml in 10 mM Tris-SO_4, pH 7.4) was mixed on a glass plate with 0.1-ml aliquots of a geometric progressive dilution of either serum from nonimmunized rabbits or of rabbit antiserum against "structural protein" from wild-type, aerobic yeast mitochondria (Tuppy *et al.*, 1968). The sera had been diluted with 0.15 M NaCl. Sera and particles were mixed with a plastic rod and agglutination was evaluated after 5 min. [b] The hemagglutination test used in expt B was carried out essentially as described by Tuppy *et al.* (1968) except that the human erythrocytes were coated with "structural protein" from wild-type promitochondria at a ratio of 1 mg of "structural protein"/1 ml of 2.5% (v/v) erythrocyte suspension. The control and antisera had been partially purified by precipitation with 50% saturated $(NH_4)_2SO_4$ and both contained 59 mg of protein/ml.

TABLE V: Amount of Mitochondria and Promitochondria in Wild-Type *S. cerevisiae* Grown under Different Conditions.[a]

	% Homogenate Protein Represented by	
	Mitochondria (after aerobic growth)	Promitochondria (after anaerobic growth)
A. 10% glucose		
Actual recovery	3.0	3.5
From F_1-ATPase	4.0	4.2
From NADH oxidase	2.8	
From succinate dehydrogenase	4.1	3.2
Mean value	3.5	3.6
B. 0.8% glucose, plus lipids		
Actual recovery	11.3	8.6
From F_1-ATPase	15.3	9.0
From NADH oxidase	11.5	
From succinate dehydrogenase	14.2	8.7
Mean	13.1	8.8
C. 10% glucose, minus lipids		
Actual recovery	4.0	2.2
From F_1-ATPase	4.6	3.0
From NADH oxidase	3.4	
From succinate dehydrogenase	4.1	1.8
Mean	4.0	2.3

[a] Wild-type yeast was grown aerobically and anaerobically as described under Materials and Methods except that the concentration of glucose in the growth medium was varied as indicated in the table, and that in expt A and B Tween 80 and ergosterol were present also during aerobic growth of the cells. For the sake of comparison, the mitochondria were isolated under identical conditions as the promitochondria. The concentration of mitochondria and promitochondria in the cell homogenates was determined either on the basis of actual recovery or computed from enzymic measurements with the aid of the expression: specific enzyme activity of homogenate/specific enzyme activity of purified (pro)mitochondria × 100. This calculation assumed that the respective enzymes were exclusively associated with the mitochondria or the promitochondria. Only the sedimentable portion of succinate dehydrogenase was considered in expressing homogenate activity. In the recovery experiments, each sucrose gradient was overlayered with only 20–60 mg of particle protein so as to avoid losses due to overloading of the gradient.

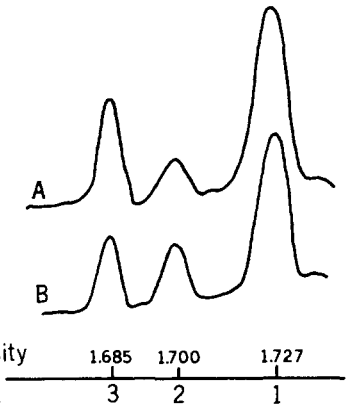

FIGURE 8: Characterization of promitochondrial DNA by equilibrium centrifugation in a cesium chloride density gradient (trace B). Microdensitometer tracings of ultraviolet photographs. The banding pattern of DNA from partially purified aerobic mitochondria is included for the sake of comparison (trace A). Peak 1 corresponds to *Pseudomonas aeruginosa* DNA which served as the reference (density = 1.727 g/cc). Peaks 2 and 3 represent nuclear and mitochondrial DNA, respectively. Both mitochondria and promitochondria were isolated from wild-type yeast cells. See Materials and Methods for further details.

drial inner membranes even under anaerobic conditions.

The Role of Promitochondria in Respiratory Adaptation. The results described thus far suggest that respiratory adaptation of anaerobic yeast cells may selectively induce the attachment of cytochromes and other constituents to the promitochondrial membranes and thus effect their transformation into functional mitochondria. If this hypothesis is correct then promitochondria isolated from yeast cells after a brief period of respiratory adaptation should contain an exceptionally high concentration of newly synthesized proteins. In an attempt to test this possibility, a lysine auxotroph of *S. cerevisiae* was grown anaerobically in the presence of a

TABLE VI: Incorporation of [^{14}C]Lysine into the Promitochondrial Fraction *in Vivo*.

	Specific Radioactivity (cpm/10 mg of protein)	
Cell Fraction	Control	Plus Cycloheximide (50 μg/ml)
Homogenate	310	<10
Soluble proteins	80	<10
"Crude particles"	870	<10
Promitochondria	1380	<10

[a] A respiratory-competent lysine auxotroph of *S. cerevisiae* was grown anaerobically, harvested in the late logarithmic phase, and pulsed with oxygen and [^{14}C]lysine as described under Materials and Methods.

limiting concentration of L-lysine and, after harvesting, simultaneously pulsed for 1 min with [^{14}C]lysine and oxygen. Under these conditions, the proteins of the isolated promitochondria were labeled almost 17 times more rapidly than the soluble cellular proteins (Table VI). In contrast, an analogous experiment with the aerobically grown cells resulted in approximately equal labeling of all subcellular fractions. It appears unlikely that the high specific radioactivity of the promitochondrial fraction was due to trapped free ribosomes containing nascent polypeptide chains since promitochondria from cell homogenates supplemented with ^{32}P-labeled yeast ribosomes contained only little ^{32}P.

The data of Table VI also indicate that the *in vivo* incorporation of [^{14}C]lysine was almost completely inhibited by cycloheximide. This antibiotic selectively inhibits protein synthesis mediated by the 80S cytoplasmic ribosomes and does not affect the mitochondrial protein-synthesizing system (Clark-Walker and Linnane, 1966). The essentially complete inhibition of promitochondrial labeling therefore suggests that the great majority of the proteins synthesized during the early phases of respiratory adaptation is assembled on cytoplasmic ribosomes. This view is strengthened by the fact that chloramphenicol, a specific inhibitor of mitochondrial protein synthesis (Rendi, 1959; Mager, 1960), does not measurably affect the *in vivo* labeling of the promitochondrial fraction either in the presence or the absence of cycloheximide.

Several uncertainties have to be resolved, however, before the preferential labeling of the promitochondrial fraction can be interpreted simply in terms of mitochondrial differentiation. For one, it remains a distinct possibility that the isolated promitochondria are contaminated by membrane-bound cytoplasmic ribosomes which may carry the bulk of the newly synthesized proteins. Indeed, the RNA content of the promitochondria is in the order of 150 μg/mg of protein and thus three times as high as that of purified aerobic yeast mitochondria (Wintersberger, 1967). It should also be remembered that our conditions of oxygen adaptation simultaneously lead to a reversal of glucose repression and might trigger several different and perhaps unrelated cellular events. However, undoubtedly the greatest difficulty arises from the observation that the preferential labeling of the promitochondria was also obtained in the strict absence of oxygen. Since the distribution of radioactivity between soluble and insoluble promitochondrial proteins was identical under aerobic and anaerobic labeling conditions, it would appear that the effect of respiratory adaptation on over-all protein synthesis is rather small (*cf.* also Fukuhara, 1967b).

While the present pulse-labeling experiments thus fail to circumscribe the role of promitochondria during respiratory adaptation, they would be consistent with the hypothesis that most, if not all, of the proteins made during the initial stages of respiratory adaptation originate in the cytoplasm and that the contribution of any mitochondrial protein synthesizing system is quantitatively rather small. A similar model has earlier been proposed for the differentiation of yeast mitochondria during glucose derepression (Jayaraman *et al.*, 1966).

Discussion

The results summarized in this report indicate that anaerobic growth of *S. cerevisiae* does not arrest the synthesis of mitochondrial inner membranes but merely modifies their composition. Thus, the mitochondrial membranes present in the anaerobic cells lack an integrated electron transfer chain and, presumably, a functional oxidative phosphorylation system. On the other hand, they have still retained such characteristic inner membrane constituents as mitochondrial ATPase (F_1) and the factor conferring oligomycin sensitivity to F_1 (Racker, 1963). It is striking that these are among the components which have earlier been shown (Kagawa and Racker, 1966) to be indispensable for the *in vitro* reconstitution of cytochrome-deficient mitochondrial inner membranes.

Our experiments also suggest that promitochondria may contain cytochrome b_1 as well as succinate and NADH dehydrogenase. However, these redox components are not unequivocal markers for mitochondrial membranes (*cf.*, *e.g.*, Bhuvaneswaran and King, 1967) and could well be associated with contaminating non-mitochondrial particles. Nevertheless, it is tempting to speculate that "cytochrome b_1" is actually cytochrome b_5 associated with the outer membrane of yeast mitochondria. This possibility is underscored by the observation that the two minor α bands detected in spectra of the aerobic "petite" mitochondria (Figure 5B) are identical in position with the α bands of cytochrome b_1. This would suggest that the mitochondrial outer membrane is still present in the anaerobic yeast cells. Alternatively, "cytochrome b_1" could be a component of microsomal membranes which are present in anaerobic yeast and which could contaminate our promitochondrial fractions (Schatz, 1963; Schatz and Klima, 1964).

The present study confirms the observation by others (Morpurgo *et al.*, 1964; Lukins *et al.*, 1966; Wallace *et al.*, 1968) that the succinate dehydrogenase content and the cytology of anaerobically grown *S. cerevisiae* are profoundly affected by the composition of the growth medium. This point is developed further in the accompanying paper which describes the lipid composition of the anaerobic yeast cells grown under different conditions (Paltauf and Schatz, 1969). It should be emphasized, however, that membranes carrying oligomycin-sensitive F_1 are still present in yeast cells which have been grown under conditions most unfavorable for mitochondrial development, *viz.*, a combination of anaerobiosis, high glucose concentration, and a lipid-deficient culture medium. It thus appears that viable yeast cells always contain mitochondrial inner membranes.

In view of the cytochrome deficiency of the promitochondrial membranes and their unusual fatty acid composition (*cf.* Paltauf and Schatz, 1969), a study of their ultrastructure will be of considerable interest. After positive staining with potassium permanganate or osmium tetroxide, the isolated promitochondria appear as small vesicles (0.2–0.6-μ diameter) without any distinctive morphological features (J. Klima and G. Schatz, unpublished data). While negative staining procedures are usually more useful for identifying mitochondrial inner

membranes (*cf.*, *e.g.*, Cunningham and Crane, 1965), they unfortunately do not consistently reveal the characteristic knob-like projections along the inner membrane of yeast mitochondria (A. Stockinger and G. Schatz, unpublished data). It is thus difficult to use negative staining for documenting the mitochondrial nature of the promitochondrial membranes. However, as described in the third paper of this series (Plattner and Schatz, 1969), we have successfully visualized promitochondria *in situ* with the freeze–etching procedure developed by Moor and Mühlethaler (1963).

The presence of incomplete mitochondria in anaerobic yeast cells is in harmony with the concept of mitochondrial continuity and is also compatible with the large body of evidence documenting the existence of a specific mitochondrial genetic system. Indeed, the observation that promitochondria contain mitochondrial DNA and are altered by the "petite" mutation raises the possibility that at least a part of this genetic system is still functional in the anaerobic yeast cells (*cf.* also Carnevali *et al.*, 1966; Fukuhara, 1967a; Rabinowitz *et al.*, 1967). Since molecular stability is one of the most intrinsic features of DNA, the presence of mitochondrial DNA in the isolated promitochondria strongly suggests a physical continuity between these particles and the fully functional mitochondria of aerobic yeast cells. Nevertheless, further work will be required to show that the anaerobic particles described here can indeed be transformed into respiring mitochondria.

Regardless of these remaining uncertainties, there appears to be little doubt that anaerobic propagation of yeast cells fails to induce a complete loss of the mitochondrial organelles. This in turn suggests that oxygen adaptation of the anaerobic cells involves the addition of electron and energy transfer components to undifferentiated mitochondrial membranes which are thereby transformed into aerobic mitochondria. In any case, there is no longer any reason to sustain the notion that respiratory adaptation of yeast is dependent upon *de novo* formation of the mitochondrial structures.

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