BIOGENESIS OF MITOCHONDRIA

XIII. The Isolation of Mitochondrial Structures from Anaerobically Grown *Saccharomyces cerevisiae*

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ABSTRACT

Morphologically intact structures have been isolated from anaerobically grown yeast cells which have many of the properties of yeast mitochondria. The structures are about 0.5 μ in diameter and contain malate dehydrogenase, succinate dehydrogenase, oligomycin-sensitive ATPase, and DNA of buoyant density 1.683 g/cc, characteristic of yeast mitochondria. The morphology of the structures is critically dependent on their lipid composition. When isolated from cells grown anaerobically in the presence of supplements of unsaturated fatty acid and ergosterol, their unsaturated fatty acid content is similar to that of mitochondria from aerobically grown cells. These lipid-complete structures consist predominantly of double-membrane vesicles enclosing a dense matrix which contains a folded inner membrane system bordering electron-transparent regions which are somewhat different from the cristae of functional mitochondria. In contrast, the structures from cells grown without lipid supplements are much simpler in morphology; they have a dense granular matrix surrounded by a double membrane but have no obvious folded inner membrane system within the matrix. The lipid-depleted structures are very fragile and are only isolated in intact form from protoplasts that have been prefixed with glutaraldehyde

INTRODUCTION

The facultative anaerobe *Saccharomyces cerevisiae* provides a versatile system for the study of the biogenesis of mitochondria. Under aerobic conditions this organism forms mitochondria which are classical in their properties, but the number, morphology, and enzyme activity of these mitochondria are also affected by catabolite repression (Ephrussi et al., 1956; Yotsuyanagi, 1962 a, b; Linnane, 1965; Jayaraman et al., 1966). Furthermore, under anaerobic conditions the organism lacks cytochromes and cannot respire but regains these characteristics when aerated (Ephrussi and Slonimski, 1950; Slonimski, 1953; Lindenmeyer and Estabrook, 1958; Wallace and Linnane, 1964; Linnane, 1965).

A fundamental problem related to the biogenesis of mitochondria is to establish the nature of the mitochondrial precursors in anaerobically grown yeast cells and to follow their development to functional mitochondria during the aeration of cells (Wallace and Linnane, 1964). Since the synthesis of ergosterol and unsaturated fatty acids is oxygen-dependent in yeast (Andreason and Stier, 1953; Bloomfield and Bloch, 1960), anaerobically grown yeast cells may be depleted of these lipid components by anaerobic growth (Jollow et al.,

1968). The cytology of anaerobically grown yeast cells is profoundly affected by both lipid-depletion and catabolite repression (Wallace et al., 1968). There is general agreement that cells grown anaerobically with lipid supplements contain mitochondrial profiles, but the nature of the mitochondrial precursors in lipid-depleted anaerobically grown cells has remained uncertain (Morpurgo et al., 1964, Tustanoff and Bartley, 1964; Wallace and Linnane, 1964; Wallace et al., 1968.) Although Wallace et al. (1968) observed vague outlines of mitochondrial dimensions in potassium permanganate-fixed, lipid-depleted anaerobic cells, membranous mitochondrial profiles were not demonstrable. More recently Damsky et al. (1969) have confirmed these observations and extended them to show that in glutaraldehyde-fixed, partially disrupted cells mitochondrial precursors are recognizable. Also employing the technique of freeze-etching, Plattner and Schatz (1969) have reported structures in glucose-repressed, lipid-depleted cells which they describe as complete with cristae and similar in appearance to the aerobic mitochondrial structures. However, it should be pointed out that these cells still contained 14–19% unsaturated fatty acid (Paltauf and Schatz, 1969), whereas in our hands viable anaerobic cells containing only 5–7% unsaturated fatty acid have been routinely obtained.

This communication describes the isolation of morphologically intact mitochondrial structures from lipid-supplemented and rigorously lipid-depleted cells. The structures from the two types of cell are markedly different in morphology and fatty acid composition, but both contain mitochondrial DNA and a number of mitochondrial enzymes.

METHODS

Growth of Cells

A diploid strain of *Saccharomyces cerevisiae* employed in previous experiments was used for the investigation and was grown in a 0.5% yeast extract-salts medium as described by Wallace et al. (1968). Glucose (5%) or galactose (5%) was used as carbon source anaerobically, and glucose at 1% as carbon source for aerobic growth. In lipid-supplemented media, ergosterol and Tween 80 were added to give concentrations of 20 mg and 5 g per liter, respectively. Cells were cultured under four conditions: (*a*) grown to 0.8 mg dry weight/ml anaerobically in lipid-limited glucose medium to give lipid-depleted catabolite-repressed anaerobic cells, designated An-Glu; (*b*) grown to 3–4 mg dry weight/ml anaerobically in glucose medium with lipid supplements to give lipid-supplemented catabolite-repressed anaerobic cells, designated An-Glu + T + E; (*c*) grown to 3–4 mg dry weight/ml anaerobically in galactose medium with lipid supplements to give lipid-supplemented partially catabolite-repressed anaerobic cells designated An-Gal + T + E; (*d*) grown to 2.0 mg/ml dry weight aerobically in glucose medium to give catabolite-derepressed aerobic cells with a normal lipid composition designated Aer-Glu.

Preparation of Protoplasts

Protoplasts were prepared by a modification of the method of Duell et al. (1964) as described by Lamb et al. (1968).

Prefixation of Protoplasts

Protoplasts were incubated for 10 min at 0°–4°C with 1% glutaraldehyde in 0.9 M sorbitol, 2 mM EDTA, pH 7.4, and then washed twice with 0.9 M sorbitol, 2 mM EDTA before being broken in a French Press in 0.5 M sorbitol, 2 mM EDTA, pH 7.4 (SE buffer).

Isolation of Mitochondrial Structures

During the isolation of mitochondrial structures from anaerobically grown cells a number of precautions were taken to prevent aerobic induction of mitochondrial components: (*a*) cells were chilled in ice to 0°C prior to harvesting and maintained at 0°–4°C throughout the isolation procedure, except for the incubation with snail enzyme; (*b*) cell suspensions and all solutions were deoxygenated by continually flushing with oxygen-free nitrogen; (*c*) cycloheximide (10 μg/ml) was added to the cells immediately before harvesting and to all solutions. Cycloheximide inhibits protein synthesis by yeast cytoplasmic ribosomes and prevents the aerobic induction of mitochondrial enzymes (Fukuhara, 1965).

After treatment of the anaerobically grown cells with snail enzyme, about 40% of the cells could be broken by osmotic shock, but about 90% of the cells could be broken by passage through a French Press at the low pressure of 500 psi. in a medium containing 0.5 M sorbitol, 2 mM EDTA, pH 7.4. Since mitochondria from aerobically grown cells had good respiratory control after the latter treatment, this method of breakage was used in all experiments. Cell debris, cells, and unbroken protoplasts were removed by three centrifugations at 1000 *g* for 10 min. The supernatant was centrifuged at 20,000 *g* for 20 min to sediment the mitochondrial fraction, which was then suspended in 50% sorbitol.

The mitochondrial fraction was layered onto a discontinuous gradient consisting of 6 ml of 80%

sorbitol and 3 ml each of 70, 60, 57.5, 55, 52.5, and 50% sorbitol in 0.02 M Tris-HCl, pH 7.4, and centrifuged at 25,000 rpm. for 2.5 hr in a Spinco SW 25 rotor (Beckman Instruments, Inc., Palo Alto. Calif.). In all cases the main fraction formed a band at a density of 1.18–1.20 g/cc which was collected for further investigation.

Electron Microscopy

Samples were treated with 1% glutaraldehyde for 30 min, diluted with SE buffer, and centrifuged at 50,000 *g* for 15 min. After two further washes, the samples were treated with OsO_4 at 1% in Veronal-acetate buffer, pH 7·2, for 2 hr, and then centrifuged in a Beckman microfuge for 10 min at full speed. The pellets were dehydrated in acetone and embedded in Araldite. Sections were cut on an LKB ultratome (LKB Instruments, Inc., Rockville, Md.) with glass knives, collected on carbon-coated grids and stained with uranyl nitrate-lead citrate for 15 min. Micrographs were examined in a Hitachi HU-11A electron microscope.

Enzyme Assays

Succinate dehydrogenase activity was measured by the method of Arrigoni and Singer (1962), malate dehydrogenase as described by Vary et al. (1969), and ATPase activity by the method of Pullman and Monroy (1963).

DNA Measurement

Particles were suspended in standard saline citrate (SSC) and lysed with sodium dodecyl sulphate at 1.3% (Schildkraut et al. 1962). The sample was treated with pronase and dialyzed overnight against SSC at 37°C. The contents of the dialysis sac were incubated with ribonuclease at 37°C for 30 min, and deproteinization was carried out as described by Wake (1967). Cesium chloride gradient centrifugation and the calculation of buoyant density was carried out as detailed by Schildkraut et al. (1962). DNA from *Micrococcus lysodeikticus*, buoyant density 1.731, was used as a standard.

Measure of fatty acid composition was as described by Jollow et al. (1968).

RESULTS

Mitochondria from Aerobically Grown Cells

We report here that yeast mitochondria isolated from aerobically grown cells can assume a number of very different conformational forms. It is important to recognize these forms in order to make a useful comparison between the mitochondrial structures isolated from aerobic and anaerobic cells.

Electron micrographs of isolated mitochondria from aerobically grown yeast (Aer-Glu) cells are shown in Fig. 1. Freshly isolated mitochondria (Fig. 1 *a*) exist in a number of morphologically distinct forms which are shown at high magnification in Figs. 1 *c–e*. The different conformational forms resemble at their extremes the so-called orthodox (Fig. 1 *c*) and condensed (Fig. 1 *e*) forms of rat liver mitochondria described by Hackenbrock (1966, 1968 *a*, *b*). The orthodox conformation (Fig. 1 *c*) is characterized by long, narrow cristae and an expanded matrix which occupies most of the mitochondrial volume, whereas the condensed conformation has large, irregular-shaped, intracristal spaces and a highly condensed matrix (Fig. 1 *e*). However, most of the freshly-isolated mitochondria appear to have a conformation which is intermediate between these two extremes, having an irregular-shaped network of cristae and a moderately expanded matrix (Fig. 1 *d*). After purification on a sorbitol gradient, most of the mitochondria are in the condensed conformation (Fig. 1 *f*). A multiplicity of conformational forms of heart mitochondria and their relationship to metabolic states have been extensively investigated by Green and co-workers (Penniston et al., 1968; Harris et al., 1968; Green et al., 1968). The nature of conformational changes in yeast mitochondria is under current investigation in this laboratory.

Mitochondrial Structures from Nonprefixed Anaerobically Grown Cells

Fig. 2 *a* shows an electron micrograph at low magnification of the particulate fraction from An-Gal + T + E yeast cells, and Fig. 3 *a*, an electron micrograph of a single structure at high magnification. The particles are clearly like mitochondria in structure, being about 0.5 μ in diameter and possessing both inner and outer membrane systems. The particles possess a granular matrix which contains electron-transparent spaces which are lined with an internal membrane. These internal spaces are reminiscent of the cristae of the condensed conformation of mitochondria from aerobically grown cells but differ in being mostly rounded in shape and only rarely forming interconnections. The structures isolated from An-Glu + T + E cells are shown in Fig. 2 *b*; they are very similar to those isolated from the An-Gal + T + E cells, but the internal membrane system and cristae are less extensively developed.

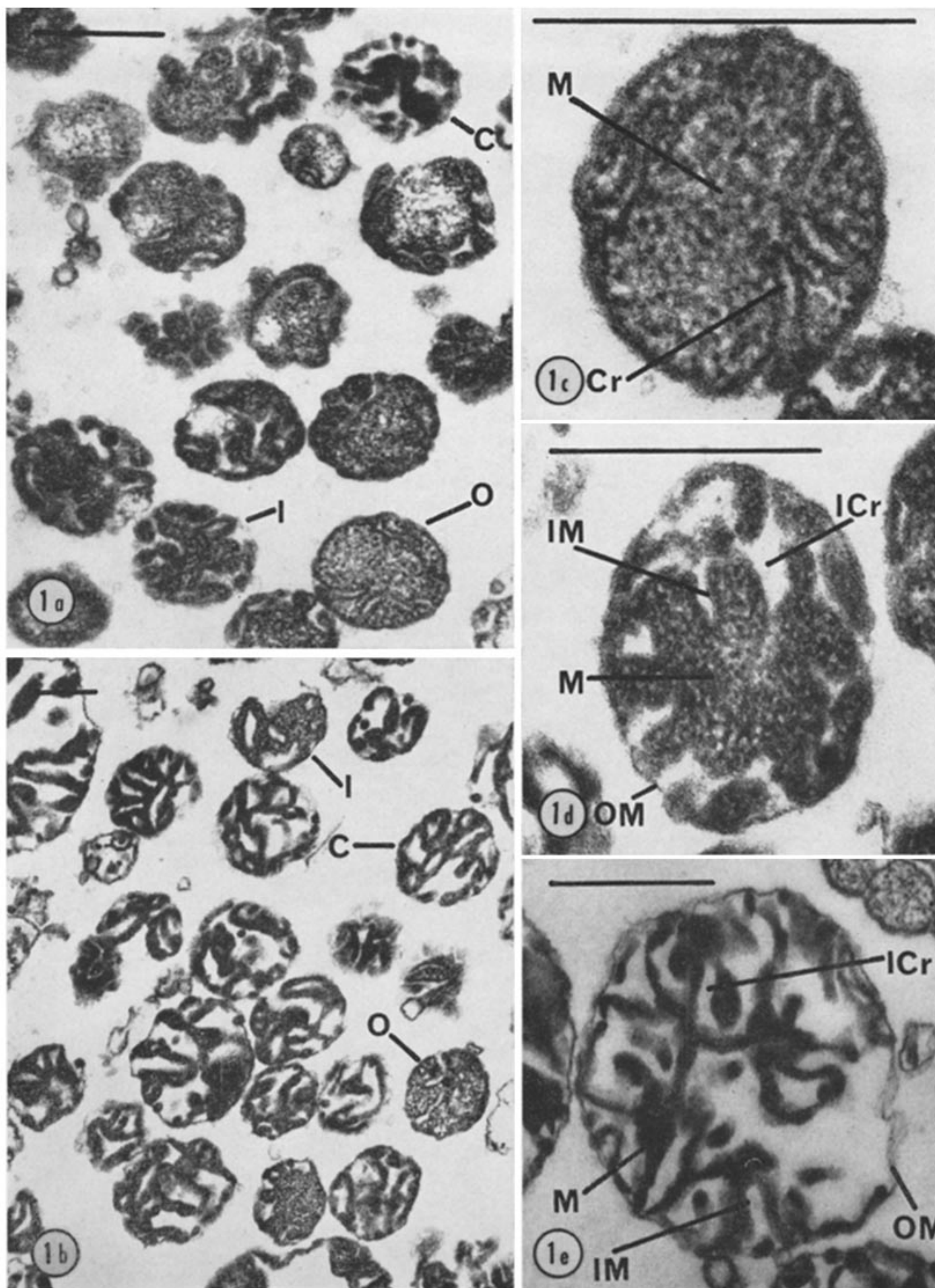

FIGURE 1 Sections through isolated mitochondria from aerobically grown cells. The bars represent 0.5 μ. Cristae (*Cr*), intracristal space (*ICr*), matrix (*M*), outer membrane (*OM*), and inner membrane (*IM*) are indicated. *a*. Freshly isolated mitochondria showing orthodox (*O*), condensed (*C*), and intermediate (*I*) conformational forms. × 39,500. *b*. Gradient-purified mitochondria. Orthodox (*O*), condensed (*C*), and intermediate (*I*) conformational forms. × 19,000. *c*. Orthodox conformational form. × 107,500. *d*. Intermediate conformational form. × 80,500. *e*. Condensed conformational form. × 50,000.

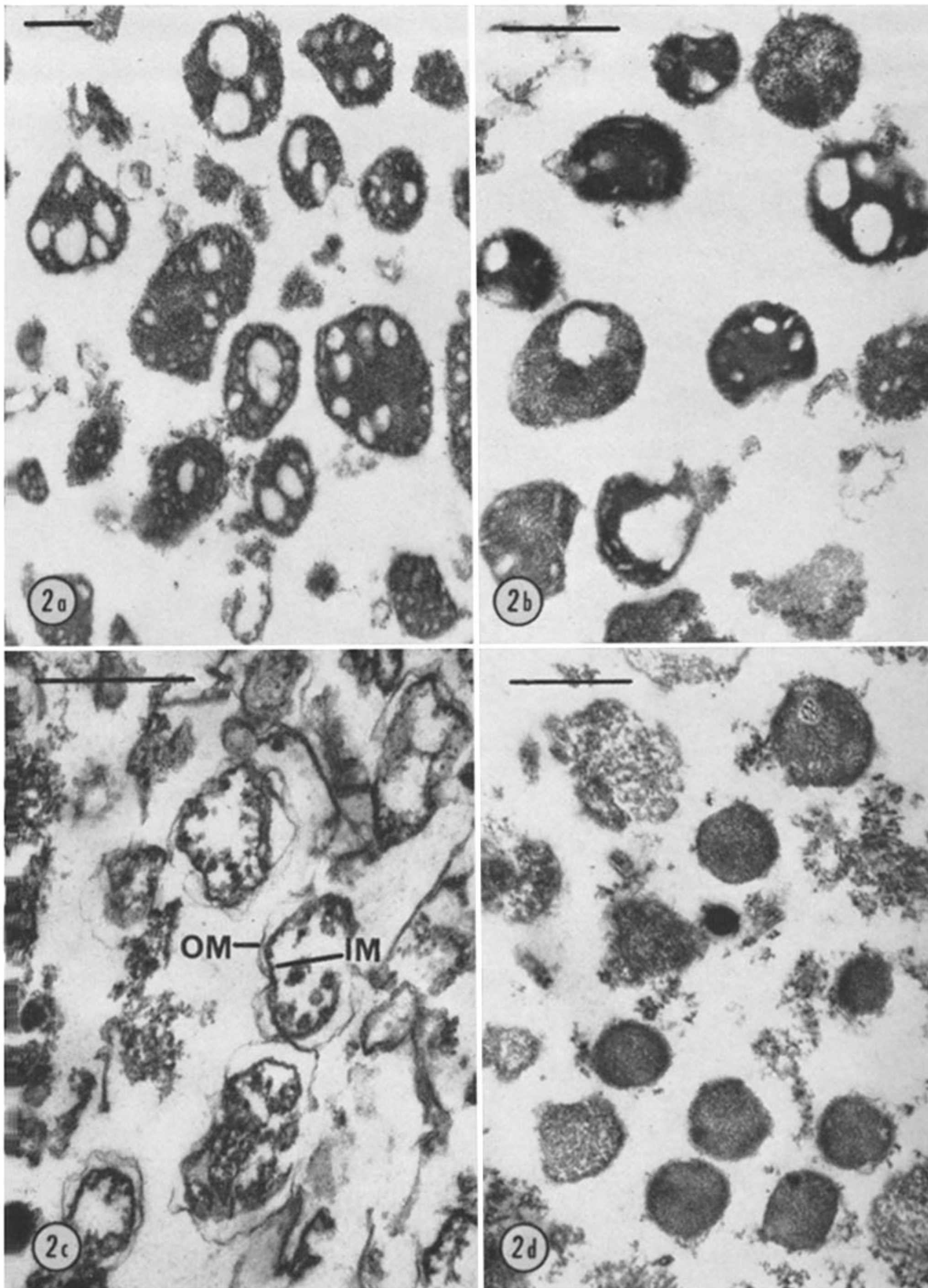

FIGURE 2 Sections through mitochondrial structures isolated from anaerobically grown cells. The bars represent 0.5 μ. *a*. Structures from nonprefixed An-Gal + T + E cells. × 20,000. *b*. Structures from nonprefixed An-Glu + T + E cells. × 34,000. *c*. Mitochondrial fraction from nonprefixed An-Glu cells. The outer (*OM*) and inner membranes (*IM*) of the disrupted structures are indicated. × 44,500. *d*. Mitochondrial structures from prefixed An-Glu cells. × 36,500.

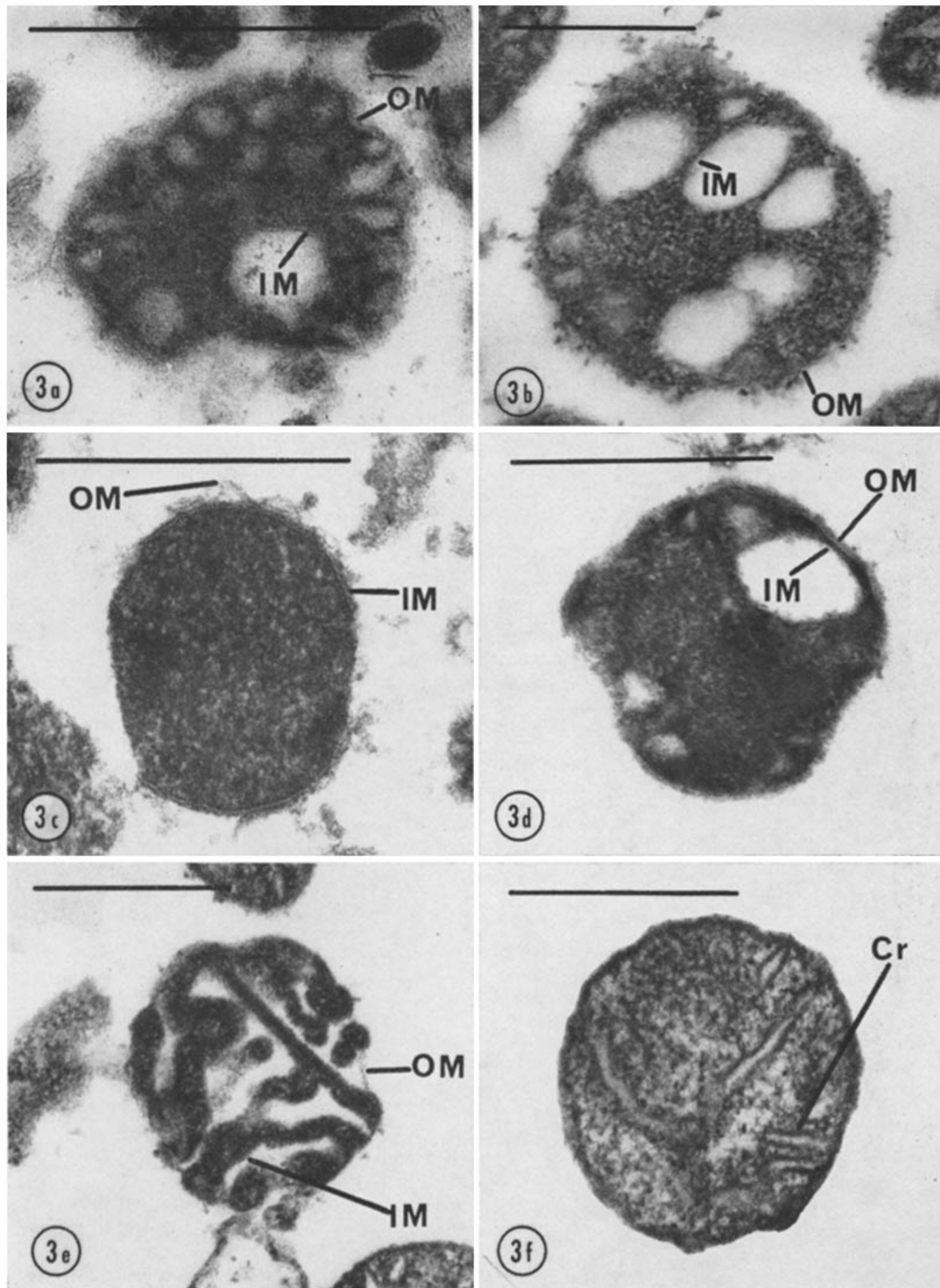

FIGURE 3 Sections through typical mitochondrial structures isolated from different cell types. Outer (*OM*) and inner membrane (*IM*) and cristae (*Cr*) are indicated. The bars represent 0.5 μ. *a*. Nonprefixed An-Gal + T + E. × 104,000. *b*. Prefixed An-Gal + T + E. × 57,000. *c*. Prefixed An-Glu. × 92,000. *d*. Prefixed An-Glu + T + E. × 77,500. *e*. Prefixed Aer-Glu, condensed conformation. × 58,000. *f*. Prefixed Aer-Glu, orthodox conformation. × 66,000.

A fraction banding at a density of 1.18–1.20 g/cc was also obtained from An-Glu cells, but Fig. 2 *c* shows that this fraction consists mainly of a disorganized mass of broken membranes. However, a few double-membrane vesicles which had broken outer membranes were also present; these appeared to have lost most of their contents, indicating that they had been badly damaged during isolation.

Mitochondrial Structures from Prefixed Cells

In order to stabilize the fragile structures, anaerobically grown, lipid-depleted cells were subjected to a mild prefixation with glutaraldehyde, a cross-linking reagent (Sabatini et al., 1963), before breaking in a French Press. It was found that incubation of the protoplasts for 10 min with 1% glutaraldehyde enabled the preservation of intact double-membrane structures. The glutaraldehyde-prefixed, lipid-depleted structures shown in Fig. 2 *d* banded at a density of 1.19–1.21 g/cc; they are vesicles about 0.5 μ in diameter which have a condensed granular matrix and are surrounded by a membrane system which at higher magnification appears to have both inner and outer membranes (Fig. 3 *c*). In contrast to the particles from the lipid-supplemented anaerobic cells, the large majority of the lipid-depleted structures have no electron-transparent regions or folded inner membrane system within the matrix, but very occasional structures showed electron-transparent regions with some indications of an inner membrane system.

Fig. 3 shows at high magnification mitochondrial structures isolated from prefixed cells, except for Fig. 3 *a* which is a micrograph of a mitochondrial profile isolated from nonprefixed An-Gal + T + E cells included for comparison. There is essentially no difference in appearance between the prefixed and nonprefixed structures from An-Gal + T + E cells (compare Figs. 3 *a* and *b*) or between structures from prefixed and nonprefixed Aer-Glu cells (compare Figs. 3 *e* and *f* with Figs. 1 *c* and *e*). However, the structure shown in Fig. 3 *c* was only seen in prefixed An-Glu cells and differs markedly from the An-Glu + T + E (Fig. 3 *d*) and An-Gal + T + E (Fig. 3 *b*) structures.

The mitochondrial nature of the structures isolated from the various anaerobically grown cells was confirmed by biochemical investigations. Table I shows that both lipid-depleted particles which contain only 5% unsaturated fatty acid and lipid-supplemented anaerobic particles which

TABLE I
Biochemical Characteristics of Mitochondrial Structures from S. cerevisiae

	Growth conditions			
Enzyme activity	An-Glu	An-Glu + T + E	An-Gal + T + E	Aer-Glu
Succinate dehydrogenase	0.7	1.4	1.7	6.5
	(0.14)	—	(0.20)	(3.6)
Malate dehydrogenase	17	72	70	150
	(8)	—	(41)	(78)
ATPase	1.7	2.0	1.9	4.0
	(0.30)	—	(0.48)	(0.60)
% Oligomycin-sensitivity of ATPase	54	68	70	75
Mitochondrial DNA	+	+	+	+
% Unsaturated fatty acid	5	80	82	82

Enzyme activities (μmoles/10 min per mg protein) were measured as described in Methods, figures in parentheses are the activities of enzymes from cells that had been prefixed with 1% glutaraldehyde for 10 min at 0°C. Growth conditions are described in Methods. The percentage inhibition of ATPase activity by oligomycin was measured at a concentration of 50 μg/mg particle protein. The mitochondrial DNA of the strain used had a buoyant density of 1.683 g/cc. The structures from the anaerobic cells contained 2.1 ± 0.5 μg mitochondrial DNA/mg protein whereas the aerobic mitochondria contained 3.0 ± 0.4 μg DNA/mg protein. Unsaturated fatty acid composition is expressed as weight per cent of total particle fatty acid.

contain about 80% unsaturated fatty acid had succinate dehydrogenase, malate dehydrogenase, and oligomycin-sensitive ATPase activities. The levels of all the enzymes in the mitochondrial structures from the anaerobic cells were considerably lower than those found in normal aerobic mitochondria, and the enzyme levels of the lipid-supplemented structures were greater than those of the lipid-depleted structures.

We have previously reported the absence of measurable succinate dehydrogenase activity in the total particulate material of lipid-depleted cells (Lukins et al., 1966). It would now appear that the high blanks encountered in these fractions rendered the assay procedures unreliable and obscured the occurrence of some succinate dehydrogenase activity.

Both types of anaerobic structure contained DNA of buoyant density 1.683 g/cc characteristic of normal respiratory-competent yeast mitochondria; contamination by nuclear DNA in these preparations was minimal as shown by the small amount of material banding at a buoyant density of 1.700 g/cc. The enzymes were identifiable in the anaerobic structures even after the glutaraldehyde treatment but were partially inactivated; malate dehydrogenase was inactivated by approximately 50%, ATPase by 75%, and succinate dehydrogenase by 90%. It is of interest that glutaraldehyde prefixation of normal aerobic mitochondria only inactivates succinate dehydrogenase by about 50%, which indicates that the enzyme arrangement within the membranes of these mitochondria is significantly different from that of the anaerobic cells.

DISCUSSION

Lipids play an important role in the structure of mitochondrial membranes (for review see Korn, 1969), and in the activity of their constituent enzyme complexes (Green and Fleischer, 1963). Moreover, unsaturated fatty acids have recently been demonstrated to have a critical function in oxidative phosphorylation by mitochondria of aerobically-grown yeast cells (Proudlock et al., 1969; Haslam et al., 1970). The present work contributes further to the understanding of the important roles of unsaturated fatty acids in the morphology of mitochondrial structures in aerobically and anaerobically grown yeast cells.

The mitochondrial structures from lipid-supplemented anaerobically grown cells contain more than 80% unsaturated fatty acid and are stable during normal isolation procedures. They have an extensively folded inner membrane system and well-developed cristae. The cristae are, however, different in appearance from those of fully developed, respiratory-competent mitochondria; they are rounded in outline and do not form the interconnected network observed in the mature organelles. Catabolite repression of mitochondrial development has some effect on the anaerobic structures, so that the structures from the lipid-supplemented, galactose-grown anaerobic cells have a more developed internal membrane system compared with the equivalent glucose-grown cells, but the structures are not radically different.

The mitochondrial structures isolated from the lipid-depleted cells contain as little as 5% unsaturated fatty acid and are very fragile. They can be stabilized by prefixation with glutaraldehyde, and consist of a dense, granular matrix enclosed within an inner and outer membrane, with no obvious folded internal membrane or cristae within the matrix.

The anaerobic mitochondrial structures are distinctly different from any of the conformations of aerobic yeast mitochondria so far reported (cf. Fig. 3). It is further recognized that the structures isolated from the lipid-depleted anaerobic cell, which show very little internal membrane organization, are the most primitive of the mitochondrial precursor structures.

The mitochondrial nature of the isolated structures is confirmed by the presence of mitochondrial DNA, and the enzymes succinate dehydrogenase, malate dehydrogenase, and oligomycin-sensitive ATPase. Similar enzymatic activities were reported in particulate fractions from lipid-supplemented anaerobically grown cells (Schatz, 1965), and more recently this was also established for particulate fractions from lipid-depleted cells (Criddle and Schatz, 1969).

We have previously reported that mitochondrial profiles are clearly recognizable in potassium permanganate-fixed, lipid-supplemented anaerobic cells whereas mitochondrial membrane was not clearly detectable in lipid-depleted cells similarly treated (Wallace et al., 1968). Differences between the lipid-depleted and lipid-supplemented cells have been further extended in the present studies on the isolated structures. These include the differences in

morphology, fragility, biochemical activities, and, significantly, the unsaturated fatty acid content, the latter possibly affecting the fixation and staining by permanganate.

The improved fixation techniques of Damsky et al. (1969), making use of glutaraldehyde, allowed the demonstration of mitochondrial structures in partially disrupted An-Glu cells. Mitochondrial structures in An-Gal + T + E and An-Glu cells have also previously been demonstrated *in situ* by Plattner and Schatz (1969), using a freeze-etching method.

The detailed changes in morphology, lipid composition, and biochemical activity of isolated mitochondrial structures accompanying the aerobic induction of anaerobically grown, lipid-depleted cells are currently being studied. The lipid-depleted organelles probably represent the most primitive form of mitochondrial precursor available for experimentation, and as such they provide an excellent model for studies on membrane synthesis and organization.

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