MITOCHONDRIA IN ANAEROBICALLY-GROWN, LIPID-LIMITED BREWER'S YEAST

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The apparent absence of mitochondria in chemically-fixed, anaerobically-grown *Saccharomyces* yeast cells (Morpurgo et al., 1964; Wallace and Linnane, 1964; Wallace et al., 1968) has resulted in a controversy over the fate of mitochondria during anaerobic growth and subsequent exposure of the cells to air. Linnane and his coworkers have shown that yeast cells grown anaerobically under lipid-

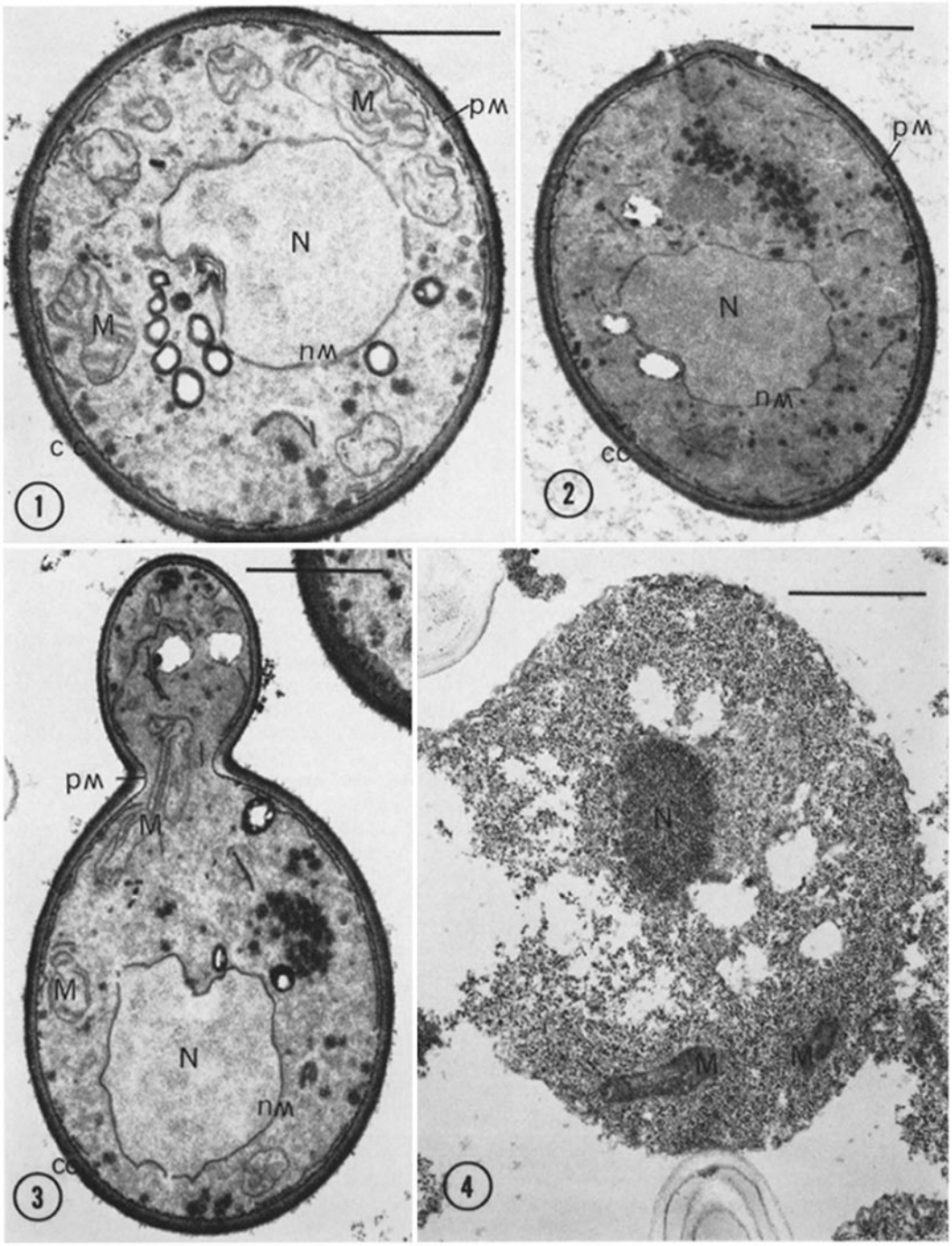


FIGURE 1 Aerobically-grown, stationary phase *Saccharomyces carlsbergensis* yeast cell. Notice well defined plasma, nuclear, and mitochondrial membranes. *N*, nucleus; *M*, mitochondrion; *cc*, cell coat; *pm*, plasma membrane; *nm*, nuclear membrane. Permanganate fixed. × 20,000.

FIGURE 2 Anaerobically-grown, lipid-limited stationary phase cell. Notice nuclear and plasma membranes. No mitochondrial profiles are visible. Permanganate fixed. × 15,000.

FIGURE 3 Anaerobically-grown, lipid-limited cell, 3 hr after the addition of air. Cell is budding, and mitochondria have appeared, indicating that cells are viable after lipid-limited, anaerobic growth. Permanganate fixed. × 20,000.

FIGURE 4 Anaerobically-grown, lipid-limited cell. Cell coat is stripped off, and cell is ruptured. Dense mitochondria can be seen in the cytoplasm. Notice very dense matrix and few cristae. Aldehyde fixed. × 20,000.

limiting conditions (Jollow et al., 1968) show no mitochondrial profiles when they are fixed with permanganate (Wallace et al., 1968), although if grown anaerobically in the presence of excess lipid, mitochondrial profiles can be observed after both permanganate fixation (Wallace et al., 1968; C. H. Damsky, unpublished observations) and glutaraldehyde fixation (Swift et al., 1968). Other fixatives such as osmium tetroxide and glutaraldehyde appear not to penetrate the intact yeast cell and show less detail than permanganate fixation (Vitols et al., 1961; C. H. Damsky, unpublished observations).

However, recent work by Schatz and his coworkers (Criddle and Schatz, 1969; Paltauf and Schatz, 1969; Plattner and Schatz, 1969) indicates that anaerobically-grown yeast contain mitochondrial DNA and oligomycin-sensitive ATPase activity. The latter is thought to be specific for the mitochondrial inner membrane (Racker, 1963; Criddle and Schatz, 1969). Furthermore, Plattner and Schatz (1969) have now demonstrated, by using the freeze-etch technique, the presence of mitochondrial-like particles, "promitochondria," in anaerobically-grown cells.

The present work attempts to reconcile this controversy. Our results indicate that permanganate fixation methods as employed by Wallace et al. (1968), Yotsuyanagi (1962), and others will not preserve mitochondria in anaerobically-grown, lipid-limited cells. Mitochondria can be observed in thin sections, however, by the alternate fixation method presented here.

MATERIALS AND METHODS

Saccharomyces carlsbergensis (ATCC 9080) cells were grown aerobically for 24 hr to a carbohydrate-starved stationary phase. Inocula were diluted 1000-fold with fresh medium which had been prepared according to Ghosh et al. (1960) and modified with 2% maltose as the carbohydrate source. The flasks were placed in an air-tight "anaerobic jar" (The Torsion Balance Co., Clifton, N.J.) which was fitted with pure hydrogen, and sealed. The cells were allowed to grow for 24 hr at 28°C, with constant shaking, and were harvested in a stationary phase in which lipids were the growth-limiting constituent.

Immediately after the jar had been opened, part of the yeast culture was poured over ice into flasks containing sufficient paraformaldehyde or glutaraldehyde solution to give a final concentration of fixative of 1 or 2.5%, respectively. The rest of the culture was poured over ice, centrifuged for less than 1 min to pack the cells, and immediately fixed with 5% permanganate (Yotsuyanagi, 1962; Avers, 1967) or fixed in 2% permanganate and poststained with uranyl nitrate as described by Wallace and Linnane (1964). The aldehyde-fixed cells were ruptured, within 20 min of initial fixation, in a Braun glass bead mill with 0.5-mm diameter glass beads for 20–30 sec. This treatment resulted in removal of pieces of cell wall and some rupturing of the cells but did not destroy the general outline of the cell (see Fig. 4). The broken cells were centrifuged at low speeds (2,000–10,000 *g* for 10 min), and fresh fixative was added (2.5% glutaraldehyde, 1% formaldehyde, or a combination of both, in 0.1 M sodium cacodylate buffer, pH 7.0). The total fixation time was approximately 3 hr. The pellets were washed with 0.1 M cacodylate buffer, pH 7.0, with 10% sucrose, with or without 1 mM uranyl acetate. The pellets were post-fixed in 2% osmium tetroxide in sodium cacodylate buffer, pH 7.0, dehydrated in ethanol, and embedded in Epon. Sections of both permanganate- and aldehyde-fixed cells were stained with saturated uranyl acetate in 50% ethanol, followed by lead citrate staining (Reynolds, 1963), and examined in an AEI 6B electron microscope.

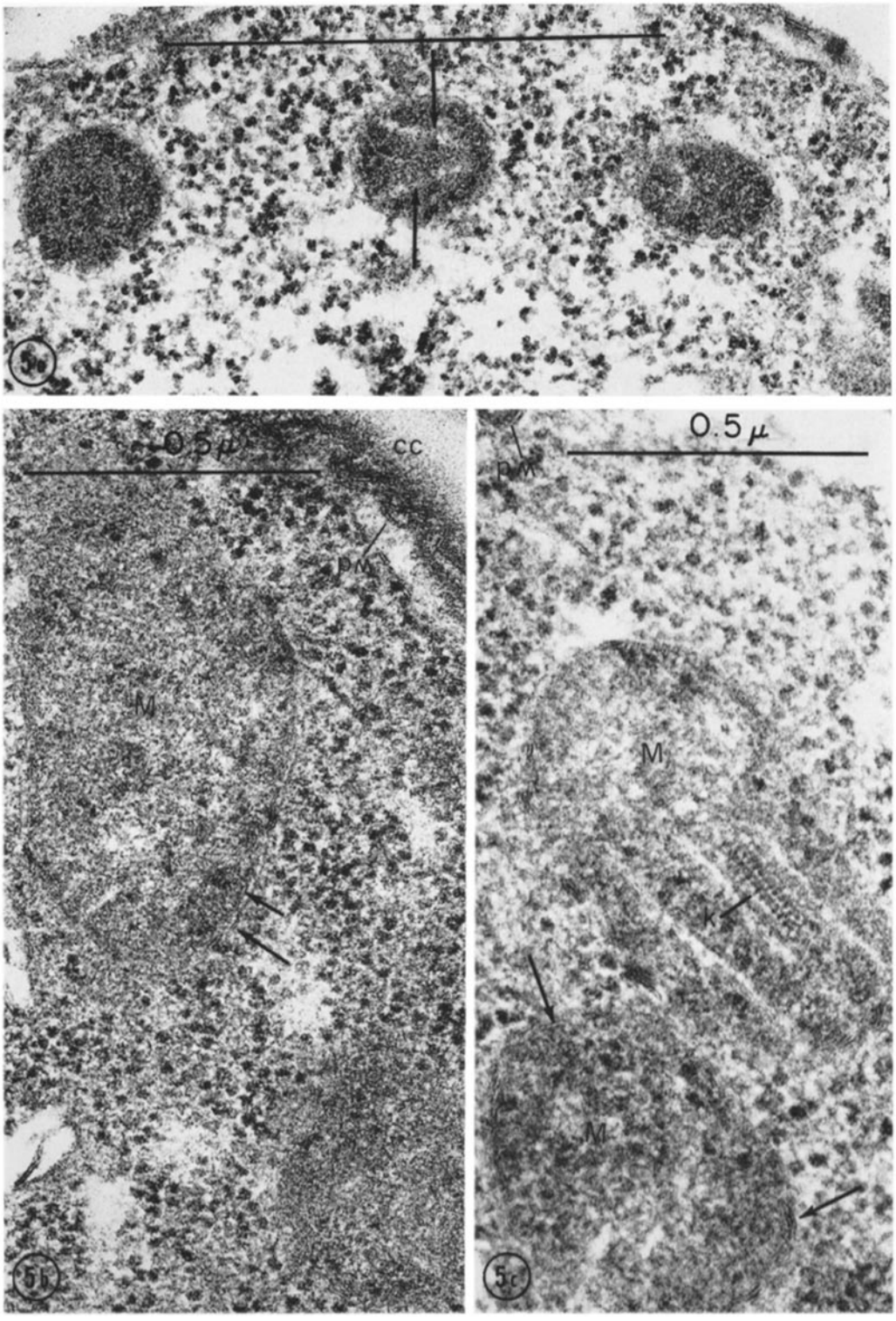
RESULTS

Fig. 1 shows a stationary phase, aerobically-grown yeast cell fixed with 5% permanganate. Nuclear and plasma membranes are well defined. Several

FIGURE 5 *a* Anaerobically-grown, lipid-limited cell. Notice very dense mitochondrial matrix. Membranes are difficult to see, and cristae appear in negative profile (arrows). Aldehyde fixed. × 75,000.

FIGURE 5 *b* Anaerobically-grown, lipid-limited cell. Notice prominent staining of plasma membrane (*pm*). Mitochondria appear noncristate in this plane of section. One and possibly two unit membrane structures can be seen surrounding mitochondrion (arrows). *cc*, cell coat; *M*, mitochondrion. Aldehyde-fixed. × 90,000.

FIGURE 5 *c* Anaerobically-grown, lipid-limited cell. Notice that membranes can be observed surrounding mitochondria in some places (arrows). Notice row of knoblike particles in long axis of one mitochondrion (*k*) (see text). Aldehyde fixed. × 90,000.



mitochondria with well developed cristae are observed in these cells.

Fig. 2 shows an anaerobically-grown, lipid-limited cell fixed with permanganate. The nuclear and plasma membranes can be seen, but they are not as well defined as those in the aerobically grown cells. Mitochondrial membranes are not visible under these conditions. This result is in agreement with the recent study by Wallace et al. (1968) of lipid-limited, anaerobically-grown cells. Most of the cells in these lipid-limited cultures appear to be viable, since after 3 hr in the presence of air, frequent budding and well-defined mitochondrial membranes can be seen (Fig. 3). Occasionally cells are seen with badly fragmented nuclei, discontinuous plasma membranes, and very low-density background cytoplasm. These are presumed to be dead cells. They comprise considerably less than 1% of the cells observed.

Fig. 4 shows a cell from the same anaerobically-grown culture but fixed immediately in 1% formaldehyde, broken in the glass bead mill, and postfixed with osmium tetroxide. The cell is ruptured in at least one place, and mitochondrial structures, measuring 0.4–0.8 μ, can be seen in the cytoplasm. They have a very dense matrix but few cristae (Figs. 4, 5). The plasma membrane can be readily observed (Figs. 5 *a–c*), but the mitochondrial membranes are very difficult to see, causing the cristae to appear in the negative (Fig. 5 *a*), similar to their appearance with aldehyde fixation alone (Sabatini et al., 1963). Mitochondrial membranes are sometimes seen and apparently consist of two unit membrane structures (Fig. 5 *b*). Occasionally a row of knoblike particles measuring about 90 A in diameter and with a center-to-center spacing of about 120 A (Fig. 5 *c*) was observed. These dimensions are similar to those of inner membrane spheres seen previously only in negatively-stained preparations of higher organisms (Fernández-Morán et al., 1964). No further attempt was made to characterize the particles. The large variation in the appearance of the mitochondria is probably due to variation in the extent of fixation. Some cells were undoubtedly ruptured more completely than others, allowing differences in the penetration of the fixative solutions. However, mitochondrial structures of some form are consistently found in the aldehyde-fixed cell.

DISCUSSION

Our results clearly demonstrate that cells can be grown under lipid-limited, anaerobic conditions so that mitochondria can be seen with aldehyde-osmium tetroxide fixation but not with permanganate fixation. We must ask, then, how these two fixative procedures differ in their staining and fixing properties and why the mitochondria of anaerobically-grown, lipid-limited cells do not stain after permanganate fixation.

Dreher et al. (1967) and Shah (1968) have demonstrated that osmium tetroxide and potassium permanganate react only with unsaturated bonds in lipid monolayers. Unless these monolayers contain cholesterol or similar compounds which contain a double bond in a ring structure, the monolayer is destroyed. Therefore, membranes such as the mitochondrial membranes of anaerobically-grown, lipid-limited yeast cells which contain very low amounts of sterol (Jollow et al., 1968; Paltauf and Schatz, 1969) would not be expected to survive fixation by permanganate or osmium tetroxide. The fact that the nuclear and plasma membranes are visible after permanganate fixation may indicate that they selectively retain the small amount of available sterol. Furthermore, Luft (1956) has shown that ribosomes and most proteins are destroyed by permanganate fixation, suggesting that the mitochondrial matrix and protein portion of the membranes would be destroyed. Therefore, the mitochondria of aerobically-grown cells may be visible only by virtue of the sterols in their membranes. Mitochondrial membranes of anaerobically-grown, lipid-limited cells would not be visible until they had acquired enough sterol to be preserved by permanganate, after the addition of oxygen (Bloch, 1965) (Fig. 3).

Aldehyde treatment preserves proteins and ribonucleoprotein by a cross-linking mechanism (Sabatini et al., 1963). Osmium tetroxide also preserves proteins by adding to their double bonds, leaving them intact and staining them. After aldehyde-osmium tetroxide treatment of anaerobically-grown, lipid-limited cells, we would expect to see mitochondria with a dense matrix and some indication of the membrane structure delineated by their fixed and stained protein portions, even though lipids might not survive the osmium tetroxide fixation.

Our results consistent with the above discussion (see Figs. 4 and 5). More work is needed to disclose the precise mechanism of fixation, and fixation

methods for observing mitochondrial morphology uniformly well under all growth conditions must continue to be improved. However, our work clearly demonstrates that under anerobic growth conditions, even in the absence of supplemental sterols, yeast cells do not lose their mitochondria completely.

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