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Development of Respiration and Mitochondria in *Mucor genevensis* After Anaerobic Growth: Absence of Glucose Repression

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Respiration and mitochondria in *Mucor genevensis*, a facultatively anaerobic dimorphic mold, have been studied in aerobically and anaerobically grown cells and in anaerobically grown cells adapting to aerobic conditions. Respiration in hyphae continues at a high level during aerobic growth but drops rapidly on exhaustion of glucose. In anaerobically grown yeastlike cells, containing no recognizable aerobic cytochromes, a small cyanide-insensitive respiration occurs. Mitochondria with well defined cristae are visible in negative contrast after $KMnO_4$ fixation of stringently anaerobic cells containing low amounts of fatty acid of which 10% or less are unsaturated. On aeration of anaerobically grown cells, respiratory capacity and cytochromes develop rapidly, even in the presence of 10% glucose, indicating that glucose does not repress development of respiration. However, mycelium formation by adapting yeastlike cells is repressed by high glucose concentration. In adapting cells, apparent changes in mitochondrial ultrastructure appear to be more related to changes in fixation properties of cells than to changes in the structure of mitochondria.

Some species of *Mucor* are of interest because of two properties: they are dimorphic and they are capable of growth under anaerobic conditions. Most recent studies have been concerned with the dimorphology of *Mucor rouxii* in particular (1, 7, 21) and have only been concerned with anaerobic growth in relation to the yeastlike development of this organism.

Little is known about the respiration of facultative anaerobic species of *Mucor*, except from the study by Terenzi and Storck (21), who suggest that yeastlike morphology and fermentation are linked and that phenethyl alcohol promotes yeastlike growth because of its uncoupling action on oxidative phosphorylation.

The ability to grow under stringent anaerobic conditions is a property peculiar to only a few fungi (20), and the most extensively studied of these is the yeast *Saccharomyces cerevisiae*. Interest in the biogenesis of mitochondria has focused on this organism because of the absence of respiration and the lack of aerobic cytochromes in anaerobically grown cells. This absence of mitochondrial components and function was seemingly substantiated by the absence of recognizable mitochondria in electron micrographs of such cells. It was believed by some that anaerobically grown cells completely lacked mitochondria under certain circumstances (14, 17, 25, 26), but this matter has now been resolved in favor of the view that poorly differentiated mitochondria or promitochondria persist even under the most stringent anaerobic conditions (6, 15, 16).

By investigating mitochondrial biogenesis in an unrelated but facultatively anaerobic organism, it was anticipated that evidence would be obtained to support the view that promitochondria persist in anaerobically grown cells and that functional mitochondria are formed from these structures. This communication reports, however, that cells of *M. genevensis*, grown under stringent anaerobic conditions, respire at a low but significant rate and contain mitochondria with recognizable cristae. Furthermore, development of increased respiratory capacity occurs rapidly upon aeration of anaerobically grown cells, even in the presence of 10% glucose, and is accompanied by changes in the absorption spectrum of whole cells.

MATERIALS AND METHODS

The organism, medium, growth conditions, and lipid analysis have been described previously (9).

Aerobic growth curve. For the measurement of respiration and growth of an aerobic culture, approximately 10^6 spores were added to 500 ml of SyO·IYE medium containing 1% glucose in a 1-liter flask. Samples of 20 ml were dispensed into 125-ml conical flasks which were then agitated on a gyratory shaker at 270 rev/min at 30 C for 12 to 14 hr. After this time, flasks were removed from the shaker at intervals and used for either the determination of dry weight or respiration. For dry weight determination, the contents of a flask were washed onto a preweighed 2.5-cm Whatman glass-fiber filter which was then dried at 110 C for 24 hr.

Adaptation conditions. Freshly harvested anaerobically grown cells were suspended in 50 ml of sterile distilled water and equal amounts were dispensed into 500-ml conical flasks, each containing 100 ml of SyO·IYE medium with 2% glycerol or the indicated concentration of glucose. The flasks were agitated at 270 rev/min on a gyratory shaker at 30 C, and samples were taken at intervals for observation in the light microscope, fixation for electron microscopy, and for determinations of respiration, absorption spectrum, and dry weight.

Glucose estimation. Glucose was estimated enzymically using glucose oxidase (Glucostat, Worthington Biochemical Corp., N.J.) after removal of cells by filtration.

Determination of respiratory rate. Oxygen uptake of washed cells was determined with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) operated at 30 C. Approximately 2 to 4 mg (dry weight) of cells was suspended in 2 ml of 0.1 M sodium phosphate buffer, *p*H 7.0, and oxygen uptake was recorded after the addition of 20 μmoles of glucose. The oxygen content of the phosphate buffer at 30 C was taken to be 0.445 microgram atom of O_2/ml (4).

After determination of the oxygen uptake, the contents of the reaction vessel were filtered onto a preweighed glass-fiber filter, and the dry weight was determined. This procedure was found to be necessary with the mycelial form of the organism which could not be uniformly dispersed. It was also found that respiratory capacity of cells declined rapidly on standing in water or phosphate buffer; therefore, respiration was determined on small samples of cells within 1 min of harvesting. When this procedure was adopted, reasonable reproducibility was obtained.

Electron microscopy. Cells were fixed in 1% $KMnO_4$ in 0.05 M sodium phosphate buffer, *p*H 7.0, for 1 hr (or for one anaerobic sample, 5 hr) at room temperature. The fixative was removed by filtration, and the cells were washed by suspension in phosphate buffer for 10 min followed by filtration and further rinsing in buffer. The cells were embedded in 2% agar and dehydrated in a graded acetone series of 10, 25, 50, 60, 70, 80, 90, 94, and 97% (v/v) for 20 min followed by 30 min in absolute acetone (3). The agar blocks were left overnight in absolute acetone and then slowly transferred to plastic by the addition of one drop of Araldite to 10 ml of acetone every 30 min. This procedure was continued for 10 hr and then the acetone was slowly removed overnight by standing the vial in a desicator over freshly heated silica gel. The blocks were placed in fresh Araldite for 24 hr and polymerized for 16 hr at 60 C.

Sections were cut with a glass knife by using a Reichert OmU2 microtome (Austria) and were picked up on naked 400 mesh copper grids. The sections were stained with lead citrate (18) for 5 min and examined in a Hitachi HU 11E electron microscope operating at 75 kv.

Absorption spectrum determination. Anaerobic and adapting cells were harvested by filtration and immediately were washed with water containing 0.05 mM sodium dithionite. Cells were suspended in 50% (v/v) glycerol -0.05 M sodium phosphate buffer, *p*H 7.0, and the absorption spectrum was determined against a starch-flour paste (10) using a Cary 14 spectrophotometer fitted with a scattered transmission accessory. The dry weight was determined as described above after filtering a sample from the absorption cell onto a preweighed glass-fiber filter.

RESULTS

Growth and respiration in aerobically cultured M. genevensis. The respiration of *M. genevensis* at different stages of the growth cycle is shown in Fig. 1. This respiration is completely inhibited by 1 mM KCN or 1 μg of antimycin A per ml. It appears that the specific rate of respiration of the organism remains constant during exponential growth but drops rapidly as the mycelium enters stationary phase. Moreover, it is apparent from the growth curve that there is no diauxic response of the organism as the glucose supply is exhausted, but only a gradual slowing of growth.

Development of respiration during aeration of anaerobically grown cells. The development of respiration when anaerobically grown cells are aerated in different media is shown in Fig. 2. The respiratory rate of actively growing anaerobic cells has consistently been found to be in the region of 10 to 15 nanogram atoms of O_2 per min per mg (dry weight), despite the fact that respiration was measured with a minimum of delay after breaking the seal of the anaerobic vessel. This respiration is insensitive to 1 mM KCN or 10 μg of antimycin A per ml.

The development of respiration, to a rate found in mycelium growing aerobically, was found to occur in 4 hr in either 1 or 10% glucose medium and in a slightly shorter time in 2% glycerol medium. Thereafter the rate of respiration remained constant in the glucose media but dropped in the glycerol medium. In 0.1% glucose medium, the rate of development of respiration was comparable initially with the rate obtained in the glycerol medium but did not increase after 2 hr, presumably because of exhaustion of the glucose from the medium.

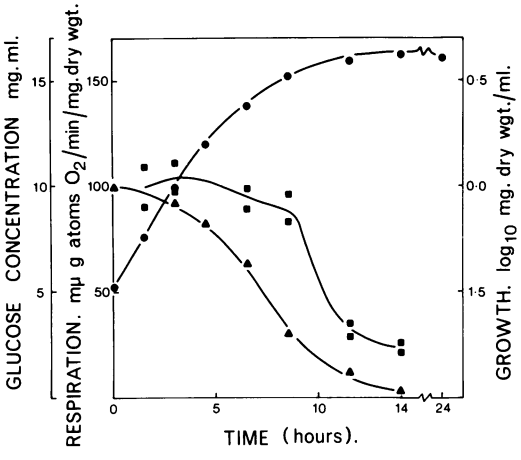

FIG. 1. *Respiration (■) and glucose concentration (▲) during aerobic growth (●) of M. genevensis in SyO·IYE medium containing 1% glucose.*

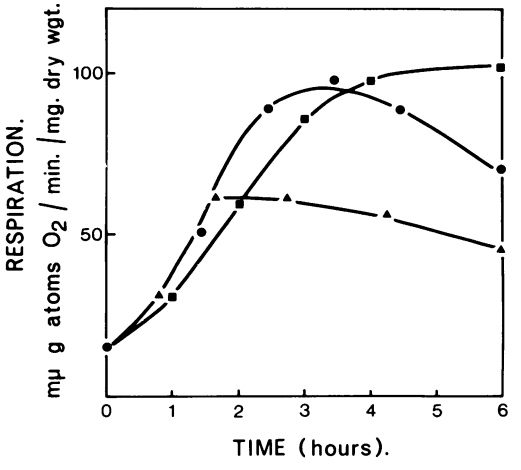

FIG. 2. *Respiration during adaptation of anaerobically grown M. genevensis in the presence of 2% glycerol (●), 0.1% glucose (▲), and 10% glucose (■). The values obtained in 1% glucose, which resemble those obtained in 10% glucose, have been omitted for clarity.*

Morphological changes associated with aeration of anaerobically grown cells. The growth of the cells during aeration in the different media is shown in Fig. 3. During the course of the experiment, there was little increase in dry weight of the cells in the glycerol or 0.1% glucose medium, whereas the dry weight of the cells in the 1 and 10% glucose media more than doubled. Despite the fact that little increase in dry weight occured in the glycerol and 0.1% glucose cultures, the majority of the yeastlike cells had initiated mycelial growth after 2 hr of incubation. This was

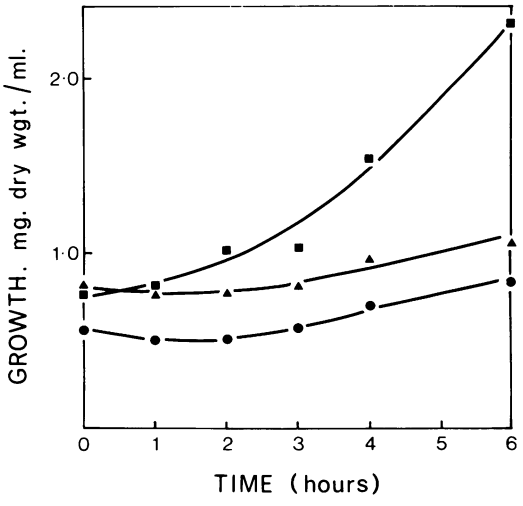

FIG. 3. *Growth during adaptation of anaerobically grown M. genevensis in the presence of 2% glycerol (●), 0.1% glucose (▲), and 10% glucose (■). The values obtained in 1% glucose, which resemble those obtained in 10% glucose, have been omitted for clarity.*

seen to occur as an elongation of preformed buds. After 4 hr, mycelium initials had grown to about two yeastlike cell diameters and after 6 hr the mycelium had developed numerous thin branches. In 1% glucose medium, only a few cells had formed mycelium initials after 2 hr of growth, but after 4 hr most cells had formed filaments. These hyphae differed in appearance from those in the glycerol and 0.1% glucose cultures in having numerous cross walls and being of a greater diameter. After 6 hr in 1% glucose, the filaments had elongated to three or four yeastlike cell diameters. The situation is different in the 10% glucose medium. No hyphae had formed after 4 hr of incubation although numerous new buds were apparent. After 6 hr of incubation, it was estimated that 2% of the cells had initiated mycelial growth.

Ultrastructure of aerobically and anaerobically grown cells. A thick multinucleate hyphal filament from an aerobically grown culture is illustrated in longitudinal section (Fig. 4). The mitochondria contain well defined double-membrane cristae which are stained darker than the surrounding dense cytoplasm. Elements of endoplasmic reticulum, vacuoles, and darkly staining bodies are also seen.

The appearance of a yeastlike cell from an anaerobically grown culture is shown in Fig. 5, and the fatty acid and ergosterol analysis of these cells shows that when ergosterol was <0.3 mg/g (dry weight) of cells and total fatty

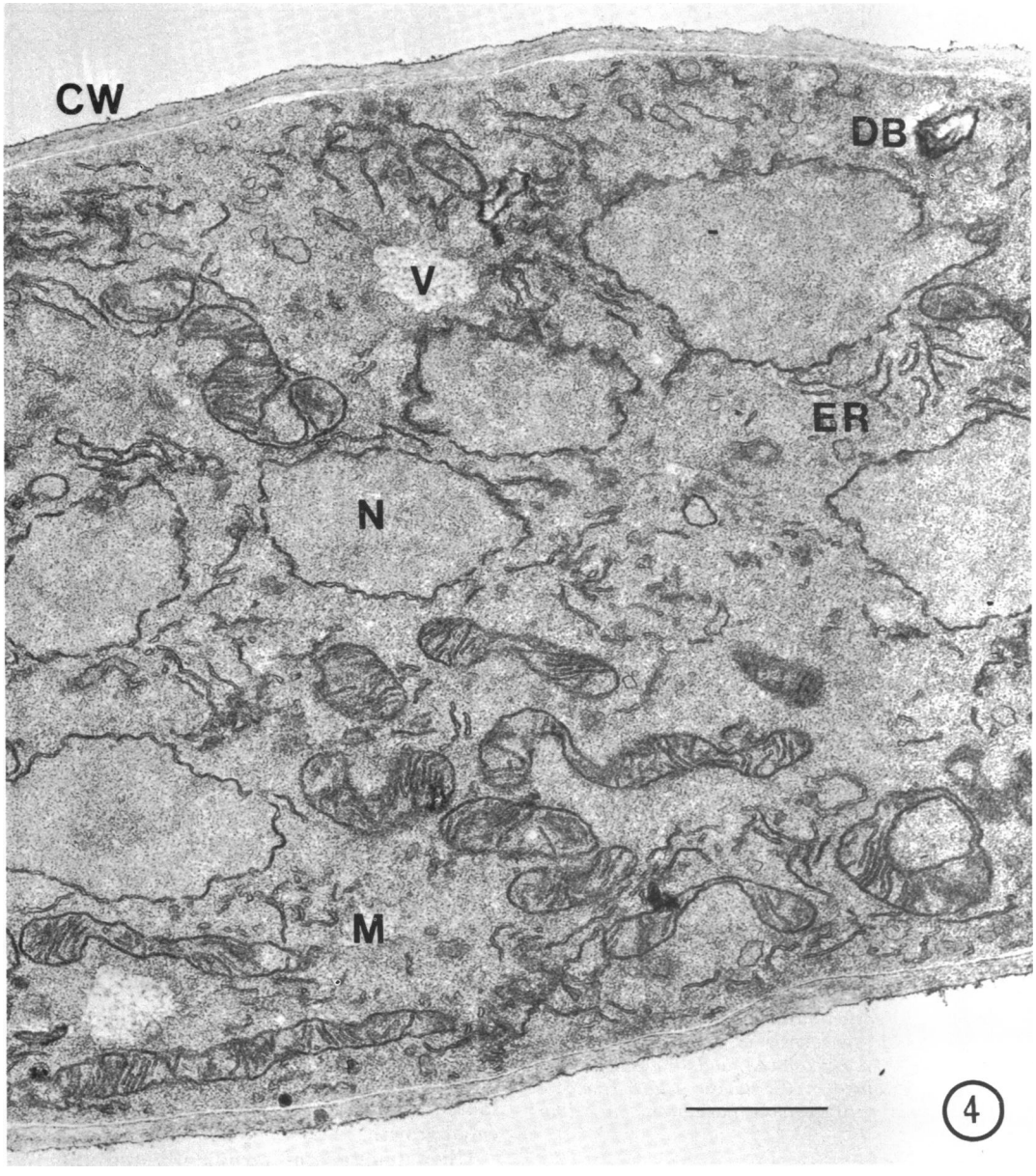

FIG. 4. *Longitudinal section through a hyphal filament of aerobically grown M. genevensis showing nuclei (N), vacuoles (V), mitochondria (M), endoplasmic reticulum (ER), cell wall (CW), and dark bodies (DB). The cells were fixed in 1% $KMnO_4$ for 1 hr. Bar represents 1 μm.*

acid was 20 and 12 mg/g (dry weight) of cells, per cent unsaturated fatty acid was 10.3 and 6.3, respectively. Corresponding illustrations are Fig. 5 and 6–9, respectively (see reference 9 for a more detailed analysis of anaerobically grown cells).

Two areas identified as nuclei (*see below*) suggest that the nuclear membrane is not preserved in anaerobic cells nor are membranes of the endoplasmic reticulum. Small dense areas are identified as mitochondria (*see below*), but these structures are only preserved in a few of the cells examined. This cell also contains vacuoles and an unstained granular substance, presumably a carbohydrate storage polymer which, although not illustrated, is also found in aerobically grown mycelium of this species and in spores of *M. rouxii* (2). The lipid analysis

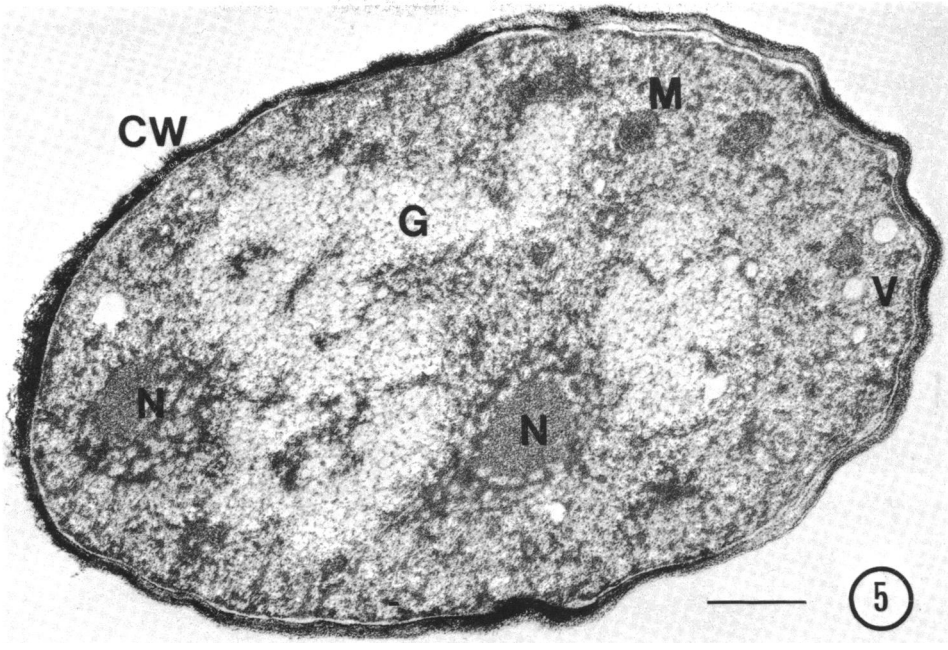

FIG. 5. *Section of a yeastlike cell of M. genevensis grown anaerobically, showing nuclear areas (N), vacuoles (V), cell wall (CW), glycogenlike granules (G), and dark areas identified as mitochondria (M). The cells were fixed in 1% $KMnO_4$ for 1 hr. Bar represents 1 μm.*

indicates that the cell contains low levels of ergosterol and fatty acid and that the unsaturated fatty acids comprise only 10.3% of the total.

The occurrence of mitochondria in anaerobic cells was confirmed by an extensive examination of thin sections. In the particular case illustrated in Fig. 6–9, the unsaturated fatty acids comprise only 6.3% of the total fatty acid of the cells. In some cells, mitochondria can be seen and they are visible in negative contrast against the densely staining background. Figure 6 illustrates the occurrence of three negatively contrasted structures close to the positively stained double-layered cell wall. In Fig. 7–9 double layered membranes, resembling in appearance the cristae of positively stained mitochondria, are apparent in these negatively contrasted structures, and it is on this evidence that these structures are identified as mitochondria.

Ultrastructure changes associated with aeration of anaerobically grown cells. The ultrastructural changes accompanying aeration of anaerobically grown cells were followed in cells suspended in 1% glucose medium. These cells initially contained 10.3% unsaturated fatty acid at the commencement of aeration, and the appearance of the corresponding anaerobic cell is illustrated in Fig. 5. In Fig. 10–12, sections of cells aerated for 1, 2, and 4 hr are illustrated. After 1 hr of aeration, the nuclear membrane is preserved during fixation, but the nuclei still resemble the nuclear areas in the anaerobic cell, which consist of an amorphous center and a granular periphery. Mitochondria appear as dense areas without any resolvable membrane system. After 2 hr of aeration, mitochondria appear as densely staining bodies and a few cristae can be seen as positively contrasted membranes against the densely staining background. The ultrastructure of cells after 4 hr of aeration is only slightly different from normal aerobic cells. Endoplasmic reticulum is now apparent and is closely associated with the cell wall in the illustrated cell. Mitochondria still appear more densely staining than in normal aerobic cells but positively contrasted cristae are now clearly visible. These cells respire at the same rate as actively growing mycelium.

Absorption spectrum changes accompanying aeration of anaerobically grown cells. The absorption spectrum of anaerobically grown cells and the accompanying changes on aeration of these cells are shown in Fig. 13. The absorption spectrum of anaerobic cells contains five bands with peaks at 623, 596, 560, 530, and 499 nm. After aeration for 1 hr, the peak at 623 nm increases, whereas the peak at

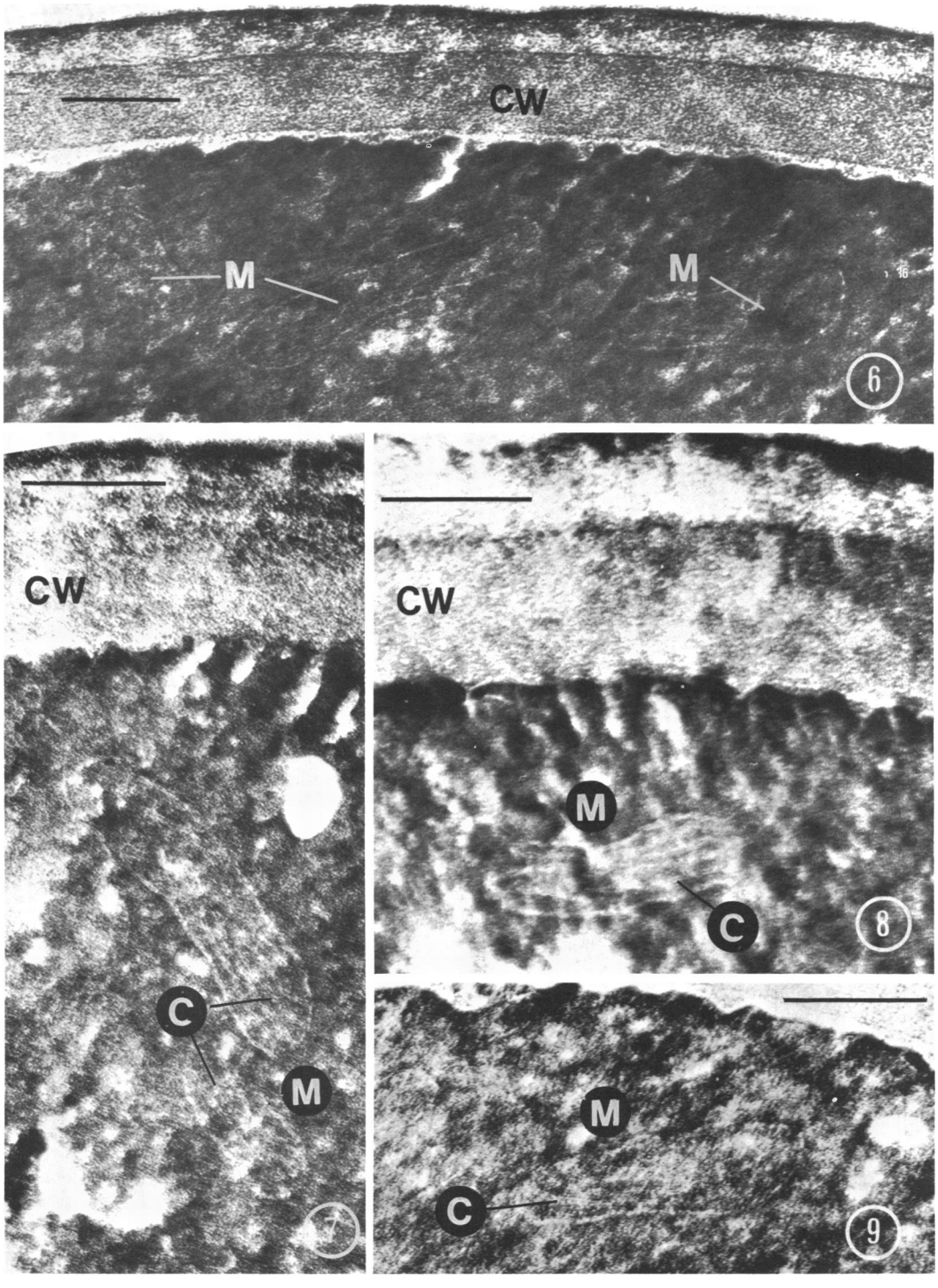

FIG. 6–9. *Sections of yeastlike cells of M. genevensis grown anaerobically showing mitochondria (M), containing cristae (C) in negative contrast and a double-layered cell wall (CW) in positive contrast. The cells were fixed in 1% $KMnO_4$ for 5 hr. Bar represents 0.5 μm.*

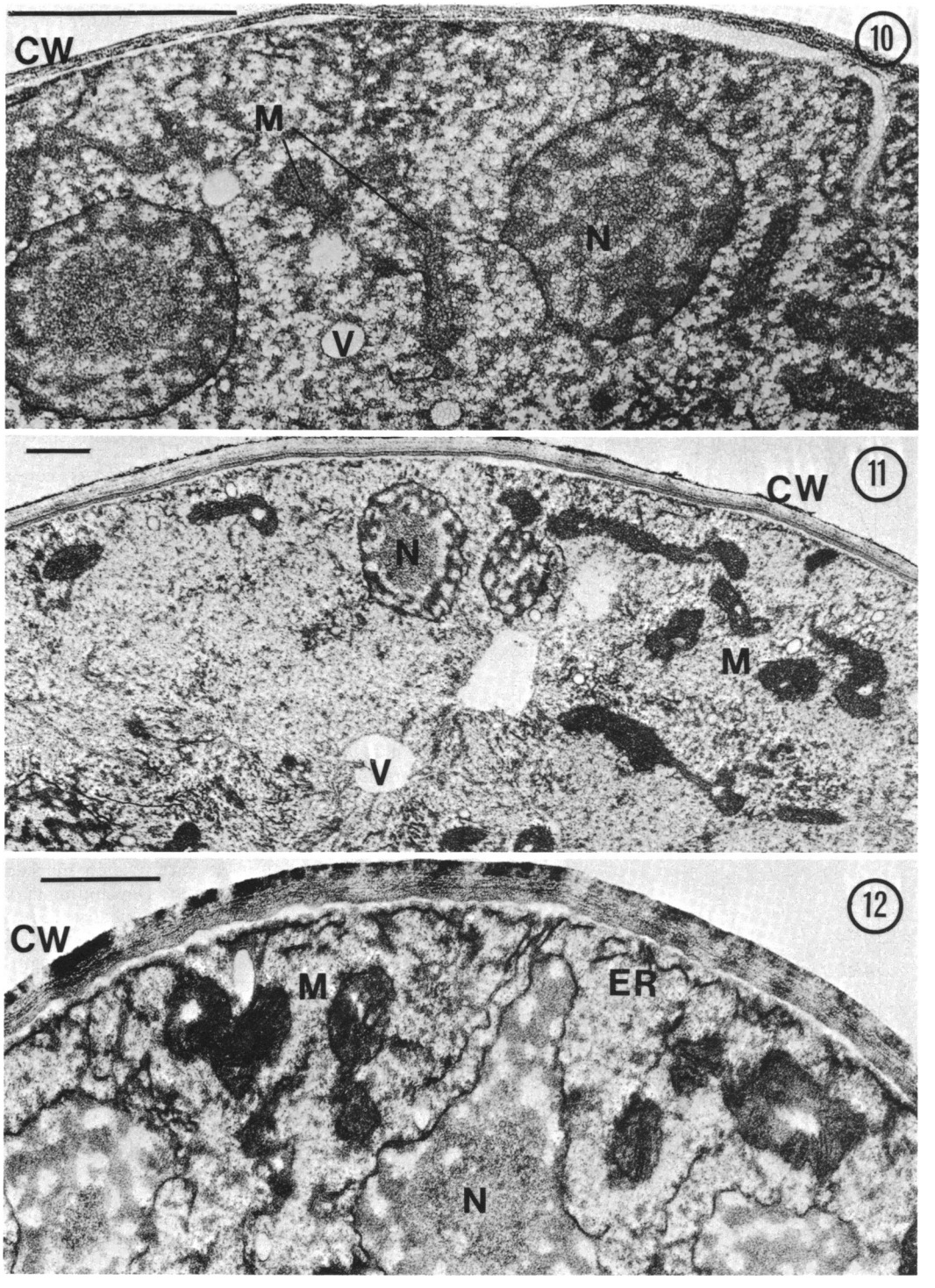

FIG. 10–12. *Sections of adapting yeastlike cells of M. genevensis 1, 2, and 4 hr after commencement of aeration, showing nuclei (N), vacuoles (V), mitochondria (M), endoplasmic reticulum (ER), and cell wall (CW). The cells were fixed in 1% $KMnO_4$ for 1 hr. Bar represents 1 μm.*

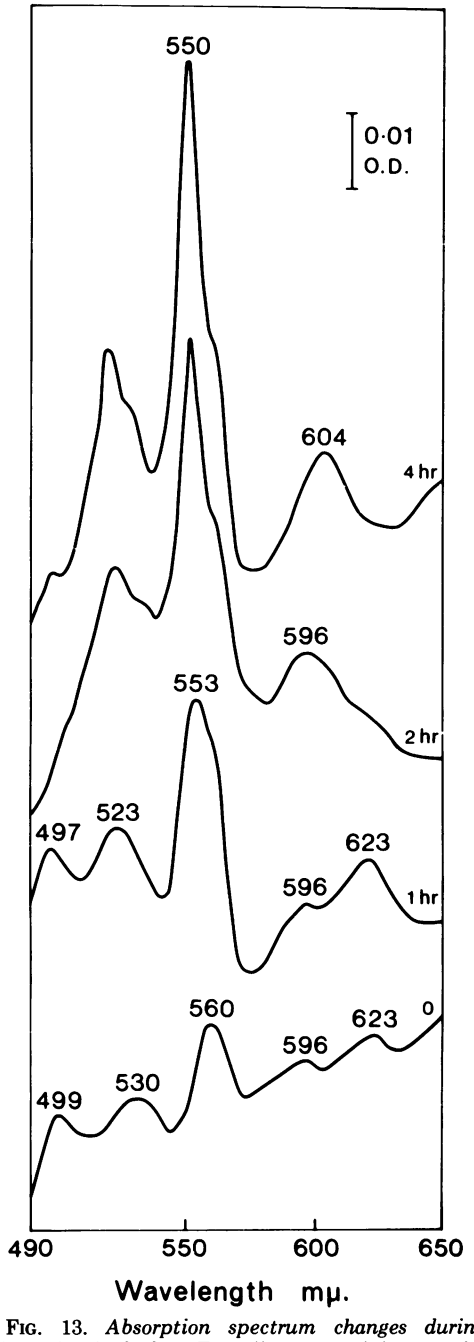

FIG. 13. *Absorption spectrum changes during adaptation in SyO·IYE medium, containing 1% glucose, of anaerobically grown M. genevensis. Cell densities in the 1-cm cuvettes were: zero hour, 35.7 mg/ml; 1 hr, 38.7 mg/ml; 2 hr, 34.1 mg/ml; 4 hr, 35.7 mg/ml.*

560 is now seen as a shoulder of a peak at 553 nm. After 2 hr of aeration, the peak at 596 nm has increased in intensity, and the appearance of a peak at 550 nm indicates that cytochrome *c* is now recognizably present. At 4 hr, the absorption spectrum is identical to that from aerobically grown mycelium with absorption peaks at 604 and 550 nm and a shoulder at 560 nm, corresponding to the absorption bands of cytochromes aa_3, *c*, and *b* respectively.

DISCUSSION

The respiration rate of aerobically growing *M. genevensis* is found to be high even in the initial part of the growth cycle when the glucose concentration is 1%. When the glucose supply is exhausted, the respiratory rate drops and no diauxic growth occurs. Conversely, with *S. cerevisiae* a definite diauxic growth curve is produced under similar circumstances (5), and respiration, which remains at a low level during the initial growth phase, increases when the glucose is exhausted (8, 12, 24).

Anaerobically grown *M. genevensis* has a low but persistent rate of respiration, despite the most stringent anaerobic growth conditions. This respiratory activity is insensitive to 1 mM KCN or 10 μg of antimycin A per ml and is not measurably present, using these inhibitors, during aerobic growth. Interestingly, anaerobically grown yeast has been reported to have a small residual respiration (11).

On aeration of anaerobically grown *M. genevensis*, there is a rapid development of respiration in glycerol or 0.1% glucose medium and a slightly slower rate of development of respiration in 1 or 10% glucose medium. This suggests that there is a small effect of glucose on the rate of respiratory adaptation but there is no effect on the final respiratory capacity in this organism. This observation is in marked contrast to results with yeast. Development of respiration is rapid in anaerobically grown yeast cells aerated in the presence of low glucose concentrations (11, 19, 22), but high glucose concentration retards the rate of formation of respiratory ability and lowers the final respiratory rate (11, 23).

The absorption spectrum of actively growing anaerobic cells of *M. genevensis* is complex, and five absorption bands are found; however, if the anaerobic cells are harvested in stationary phase, only absorption bands at 590 and 556 nm remain. The absorption spectrum of anaerobically grown *M. genevensis* is similar in some respects to that of anaerobically grown *S. cerevisiae*, but no definite conclusions can be formed in this regard since the growth medium has been shown to influence the absorption spectrum of yeast under anaerobic conditions (25).

The production of mycelium by adapting cells in glycerol or 0.1% glucose medium is

rapid, and cells suspended in 1% glucose medium also develop filaments after 4 hr of aeration. By contrast, cells suspended in 10% glucose medium continue growth by budding for at least 6 hr despite the fact that in these cells respiratory ability develops as rapidly as in cells suspended in 1% glucose medium. It appears, therefore, that glucose or products of glucose metabolism act as repressors of mycelium formation in this species and that this repression is not associated with respiration. There is a previous report of the effect of glucose in repressing hyphal morphogenesis in other species of *Mucor* (1).

The ultrastructural appearance of anaerobically grown cells of *M. genevensis* is notably different from that of aerobic mycelium when the same fixation procedure is used. Membranes of the nucleus, endoplasmic reticulum, and cell envelope are not preserved in anaerobically grown cells, whereas mitochondrial membranes are sometimes visible in negative contrast. In aerobic cells, all these membranes fix well with $KMnO_4$ and can be visualized in positive contrast against the lighter staining cytoplasm.

The anaerobic cells used for fixation in this study contain low levels of fatty acid and ergosterol, and 10% or less of the total fatty acids are unsaturated compared with about 80% unsaturated fatty acids in aerobic cells (9). This may be a significant factor in the fixation of the anaerobic cells as $KMnO_4$ has been suggested to oxidize double bonds in unsaturated fatty acids (13), and this could lead to deposition of MnO_2 at these sites.

Some mitochondria in anaerobically growing *M. genevensis* still contain numbers of cristae, whereas, in anaerobic yeast, only an occasional internal membrane is observed in promitochondria (6, 15). In yeast, glucose represses respiration and also causes alterations in the appearance of mitochondria (5). Under anaerobic conditions, therefore, two factors operate to alter mitochondrial structure in yeast, whereas, in *M. genevensis*, lack of oxygen is possibly the only significant factor.

Interpretation of ultrastructure changes which occur on aeration of anaerobically grown cells are complicated by the changes in fixation properties of the adapting cells. After 1 hr, mitochondria appear as dark areas with no recognizable membranes either in positive or negative contrast. After 4 hr of aeration, mitochondria still stain darker than in aerobically grown mycelium, but cristae are now apparent in positive contrast. This time corresponds to the appearance of a normal absorption spectrum and the development of full respiratory capacity.

Definitive evidence has recently been presented in favor of the view that respiratory adaptation of anaerobically grown yeast involves differentiation of existing promitochondria (15). The present report provides evidence that mitochondria with well differentiated cristae exist in stringently anaerobic cells of *M. genevensis*. It therefore seems probable that these mitochondria become functional during respiratory adaptation.

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