THE BIOGENESIS OF MITOCHONDRIA

II. The Influence of Medium Composition on the Cytology of Anaerobically Grown *Saccharomyces cerevisiae*

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ABSTRACT

Yeast cells grown anaerobically have been shown to vary in their ultrastructure and absorption spectrum depending upon the composition of the growth medium. The changes observed in the anaerobically grown cells are governed by the availability of unsaturated fatty acids and ergosterol and a catabolite or glucose repression. All the cells contain nuclear and plasma membranes, but the extent of the occurrence of vacuolar and mitochondrial membranes varies greatly with the growth conditions. Cells grown anaerobically on the least nutritive medium, composed of 0.5% Difco yeast extract–5% glucose–inorganic salts (YE-G), appear to contain little vacuolar membrane and no clearly recognizable mitochondrial profiles. Cells grown anaerobically on the YE-G medium supplemented with Tween 80 and ergosterol contain clearly recognizable vacuolar membrane and some mitochondrial profiles, albeit rather poorly defined. Cells grown on YE-G medium supplemented only with Tween 80 are characterized by the presence of large amounts of cytoplasmic membrane in addition to vacuolar membrane and perhaps some primitive mitochondrial profiles. When galactose replaces glucose as the major carbon source in the medium, the mitochondrial profiles within the cytoplasm become more clearly recognizable and their number increases. In aerobically grown cells, the catabolite repression also operates to reduce the total number of mitochondrial profiles. The possibility is discussed that cells grown anaerobically on the YE-G medium may not contain mitochondrial membrane and, therefore, that such cells, on aeration, form mitochondrial membrane from nonmitochondrial sources. A wide variety of absorption compounds is observed in anaerobically grown cells which do not correspond to any of the classical aerobic yeast cytochromes. The number and relative proportions of these anaerobic compounds depend upon the composition of the growth medium, the most complex spectrum being found in cells grown in the absence of lipid supplements.

INTRODUCTION

The yeast, *S. cerevisiae*, offers a biological system which is perhaps unique for the scope it affords for the study of the biogenesis of mitochondria. Grown under aerobic conditions in the absence of glucose, the organism forms mitochondria with classical properties (Agar and Douglas, 1957; Slonimski, 1953; Linnane and Still, 1955; Vitols and Linnane, 1961), but the aerobic formation of the mitochondria is also subject to a glucose (catabolite) repression affecting both the number of profiles formed and their composition (Ephrussi et al., 1956; Yotsuyanagi, 1962 *a,b*; Utter et al.,

1967; Linnane, 1965; Jayaraman et al., 1966). Indeed, strongly repressed cells may contain only primitive organelles with little capacity for aerobic oxidation, the cytochrome *a*, a_3 level of the cells approaching zero.

Anaerobically grown *S. cerevisiae* has not been so intensively studied as aerobic cells, but we have briefly reported that the anaerobic cells appear to be free of recognizable mitochondrial profiles and that the synthesis of the latter is induced by oxygen (Wallace and Linnane, 1964; Linnane, 1965). We have suggested that the presence of preexisting mitochondrial profiles is, therefore, not conditional for the synthesis of new mitochondria by these cells. However, while Polakis et al. (1964) have confirmed these observations, the situation is not so simple as it at first seemed, and it has become apparent that oxygen is not the only factor which determines the capacity of the cells to form mitochondrial profiles. Morpurgo et al. (1964) have reported the presence of mitochondrial profiles in anaerobically grown *S. cerevisiae*, provided the medium is supplemented with large amounts of ergosterol and unsaturated fatty acids. These authors have suggested that anaerobic cells without profiles are nonviable. Tustanoff and Bartley (1964) described the occurrence of mitochondrial profiles in cells grown anaerobically on a galactose medium containing unsaturated fatty acids and ergosterol.

It is apparent that there is considerable disagreement on the cytological characteristics of anaerobically grown yeast cells. In view of the importance of this information to the whole problem of the biogenesis of mitochondria, a study has been carried out on the effects of unsaturated fatty acid, sterol, and catabolite repression, separately and in combination, on the cytology of the anaerobic cell. Although the primary purpose of this investigation was the study of the mitochondria, the growth conditions employed also had striking effects on the formation of the cell vacuole and some other features of the cell, and these are described.

MATERIALS AND METHODS

Organism

A locally isolated diploid strain of *Saccharomyces cerevisiae* was used throughout the experiments. It was maintained on slopes composed of 3% malt extract, 10% glucose, 0.2% $(NH_4)_2$-HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 2.5% agar.

Growth Media

The liquid medium employed in the studies contained in each liter of distilled water:

Difco yeast extract	5 g	$CaCl_2$	0.1 g
NaCl	0.5 g	KH_2PO_4	1.0 g
$MgCl_2 \cdot 6H_2O$	0.7 g	$FeCl_3$	5 mg
$(NH_4)_2SO_4$	1.2 g	Glucose or galactose	50 g.

The medium is denoted as YE-G or YE-GAL depending upon whether glucose or galactose, respectively, was used as the carbohydrate.

In lipid-supplemented media, ergosterol and Tween 80 were added to give concentrations of 20 mg and 5 g per liter, respectively. Tween 80 (fatty acid composition: oleic 71 moles %, palmitoleic 13 moles %, myristoleic 3 moles %, and saturated fatty acid 13 moles %) served as a water-soluble source of unsaturated fatty acid.

Glucose medium supplemented, for example, with Tween 80 and ergosterol is denoted as YE-G + Tween 80 + ergosterol, and so on for other supplemented media.

Growth Conditions

Aerobically, the cells were grown at 28°C in 250 ml of medium in 1-liter conical flasks on a reciprocating shaker, or in 15 liters of medium in 18-liter containers under conditions of forced aeration.

For anaerobic growth, the inoculum was taken from an aerobic YE-G shake culture which had reached the stationary phase, and added to 1 or 15 liters of medium in an air-tight flask or carboy to give an initial cell density of about 0.02 mg dry weight of cells/ml of medium. The containers were fitted with a rubber stopper carrying gas inlet and outlet tubes. Air leakage through the stopper was prevented by tying the stopper to the flask with string and pouring molten paraffin wax around it for the purpose of ensuring a good seal. After inoculation, the medium was thoroughly flushed with oxygen-free nitrogen for 45 min, and the cells were grown at 28°C with continuous stirring by means of a magnetic stirrer. The complete removal of oxygen from the commercially available oxygen-free nitrogen was ensured by passing the nitrogen slowly through a series of solutions of vanadyl sulfate reduced with amalgamated zinc (Meites and Meites, 1948). The gas that evolved during the growth of the culture was allowed to escape through a water trap which also served to prevent any back flow of air. After the cells reached the early stationary phase of growth (16–20 hr), the culture

was poured onto a large volume of ice immediately after the flask was opened, so that the temperature of the cells and culture medium was lowered to 0–2°C within 2 min. The cells were then collected by centrifugation and washed with water, all at 2°C. These procedures are essential to prevent any oxygen-induced changes in the cytology and enzymology of the cells during manipulation in air.

For study of the effects of prolonged anaerobiosis on the organism grown on the YE-G medium, the anaerobic cultures were repeatedly subcultured under sterile anaerobic conditions. To perform the subculture we clamped the gas outlet tube of the culture flask so that the still-fermenting culture would build up a considerable pressure within the flask. When a sterile, paraffin-rinsed, air-free syringe was inserted into the gas inlet tube, the pressure within the flask could be employed to force some of its contents into the syringe. This cell suspension was then injected through the rubber bung into fresh YE-G medium which had previously been flushed with oxygen-free nitrogen. After inoculation, we flushed the medium once again with nitrogen to ensure removal of traces of oxygen. The culture was then allowed to grow under the same conditions as described above.

Electron Microscopy

We have employed potassium permanganate for the routine preservation of aerobic yeast cells, and fixation for 10–30 min has usually proved satisfactory (Wallace and Linnane, 1964). The procedure adopted for the fixation of all anaerobic cells described in this paper was as follows, and the rationale for this procedure is presented in the Discussion. Well washed cells (0.5–0.8 ml of packed cells) were suspended in 10 volumes of freshly prepared 2% potassium permanganate for 2 hr at 0°C followed by a further suspension for 3 hr in 10 volumes of fresh permanganate at room temperature. The cells were washed twice with water and then postfixed in 10 volumes of

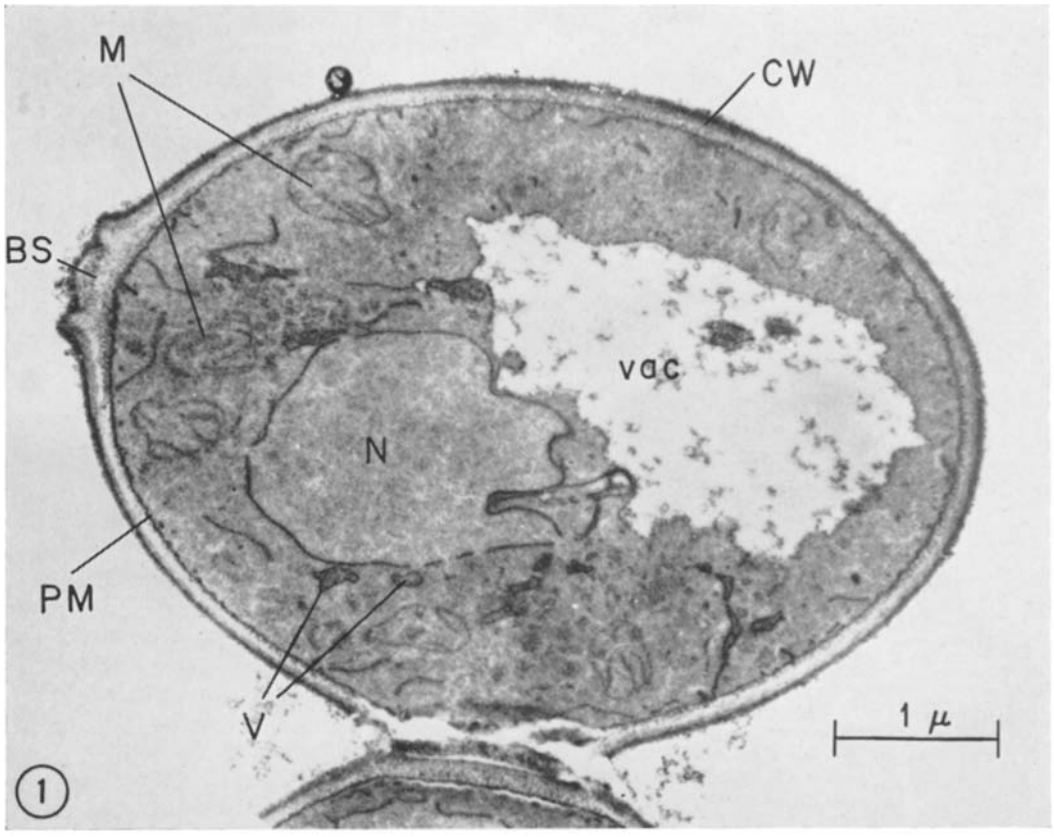

FIGURE 1 A typical section of *S. cerevisiae* grown aerobically on YE-G medium. The mitochondria (*M*), nucleus (*N*), vacuole (*VAC*), cell wall (*CW*), plasma membrane (*PM*), bud scar (*BS*), and electron-opaque vesicles (*V*) are all evident in this section. × 22,000.

a solution of 1% uranyl nitrate–1% potassium dichromate for 30 min at room temperature.

The fixed cells were washed with water, dehydrated in alcohol, and impregnated with methacrylate (three parts methyl methacrylate and seven parts butyl methacrylate) in the usual way. Embedding was completed by incubation in capsules at 50°C for 24 hr. Sections were cut on an LKB ultrotome with a diamond knife. The sections were mounted on copper grids coated with parlodion film, stabilized with a carbon layer, and the electron micrographs taken with a Siemens Elmiskop I.

RESULTS

Cytology of Aerobic Cells

The fine structure of cells of *S. cerevisiae* grown aerobically on the YE-G medium is shown in Fig. 1. A well defined nucleus, a cell wall, a plasma membrane underlying the cell wall, and a vacuole are apparent and, under these growth conditions, there are about three to eight mitochondria per cell section. In these cells, electron-opaque vesicular elements are often seen. These structures are bound by a single unit membrane enclosing some electron-opaque granular material; their nature is unknown. The ultrastructure of the aerobic cells is not detectably affected by the addition of ergosterol and/or, Tween 80 to the YE-G medium. However, growth on YE-GAL medium, or on YE-G medium modified by reduction of the glucose concentration from 5 to 1%, produces cells with the same over-all characteristics except that the number of mitochondria observed per cell section is increased to 10–20 and the vesicular elements are rarely seen (Fig. 2).

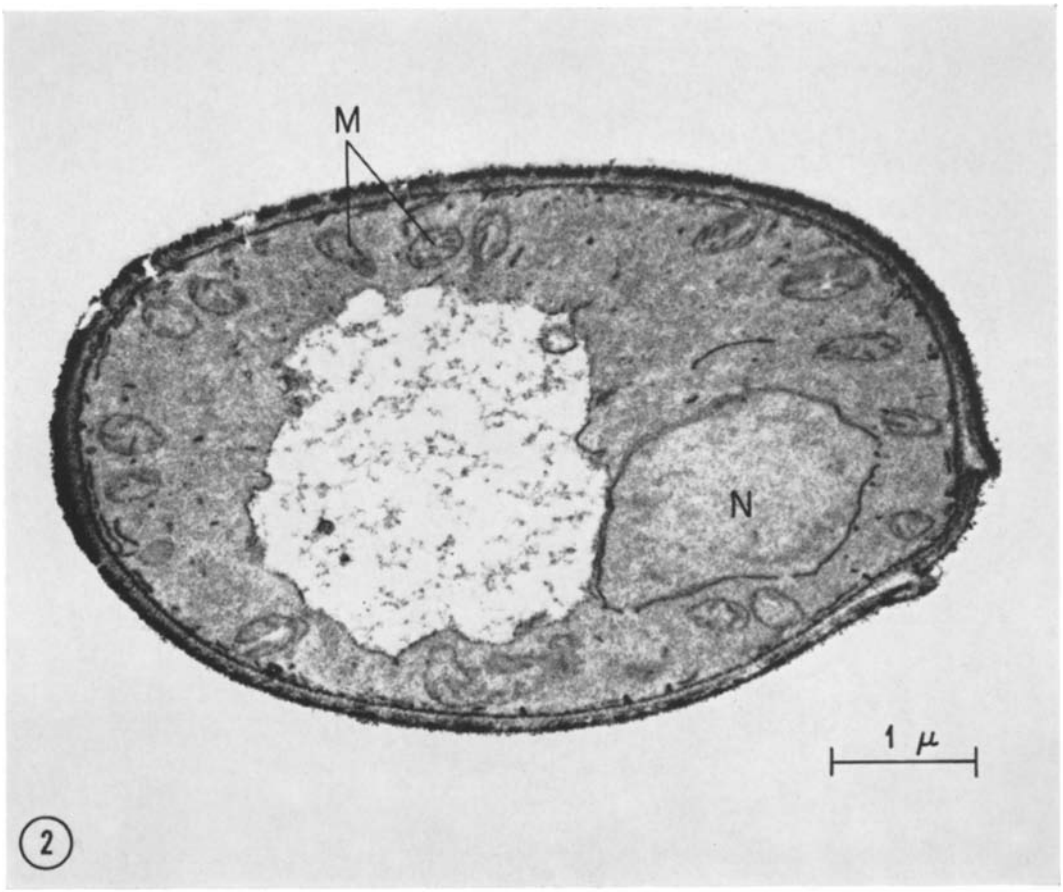

FIGURE 2 Section through a cell grown aerobically on YE-GAL medium. In comparison with Fig. 1, many more mitochondria (*M*) can be seen. × 18,000.

Cytology of Anaerobic Cells

For anaerobic growth, yeasts require an external source of unsaturated fatty acids and sterol, such as ergosterol (Andreasen and Stier, 1953, 1954). In the YE-G medium, the yeast extract serves as a growth-limiting source of these essential lipids. Thus, cells cultured anaerobically on the YE-G medium, under the conditions of the present experiments, undergo only five to six divisions per growth cycle, whereas if the medium is supplemented with additional ergosterol (20 mg/l) and unsaturated fatty acids (5 ml of Tween 80/l) the growth cycle is made up of six to eight divisions and thus the final cell yield is considerably greater. Cells grown on the YE-G medium are, in effect, lipid starved and, as described in an accompanying paper, (Jollow et al., 1968) they contain only about 2% of the level of ergosterol and unsaturated fatty acids found in aerobically grown cells. Thus, it is possible to examine the effects of unsaturated fatty acid and ergosterol, singly and in combination, on the detailed cytology of the yeast cell.

Many of the basic features of the aerobic cell are recognizable in the anaerobically grown organism irrespective of the growth medium (Figs. 3–8): viz., the cell wall, the plasma membrane, and the nucleus. However, the occurrence and appearance of some other features of the aerobic cell, including the mitochondria, are profoundly influenced by the composition of the culture medium. Again, a cytoplasmic membrane system of diverse form may be observed, but its extent and organization are likewise very much influenced by the nature of the growth medium. A detailed consideration of the effects of media composition on the cells follows.

CELLS GROWN ON THE YE-G MEDIUM: The

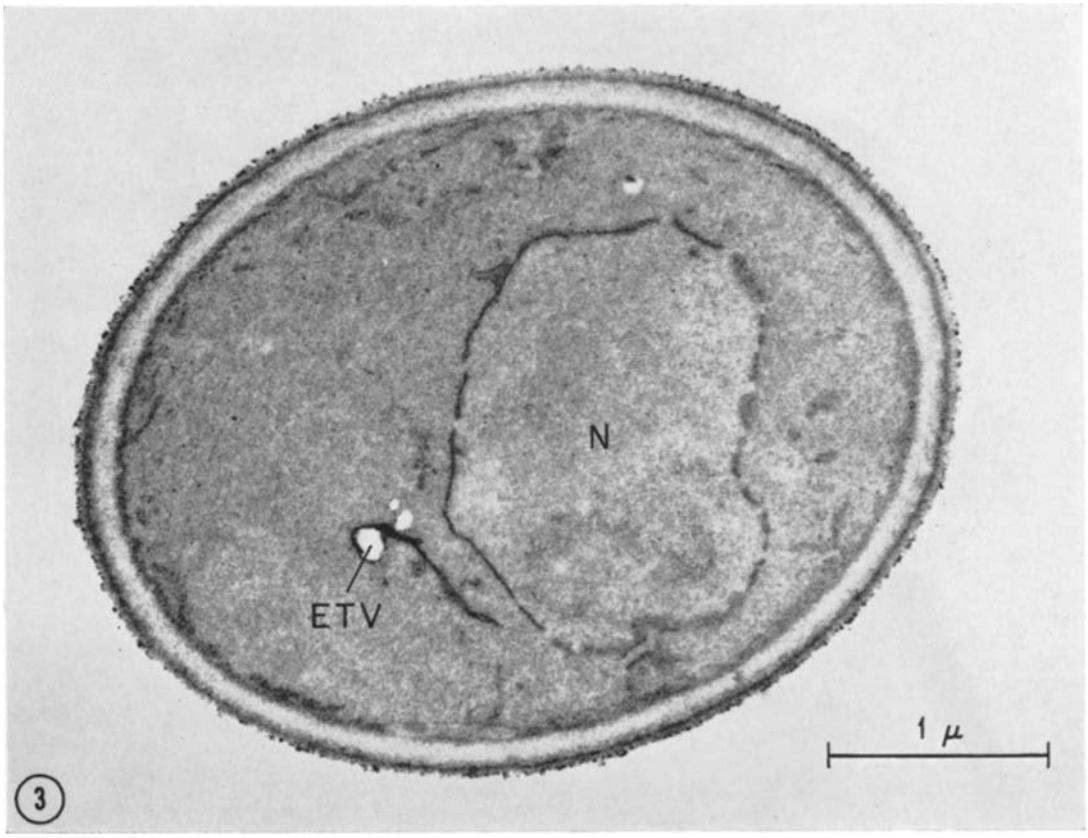

FIGURE 3 Section through a cell grown anaerobically on the YE-G medium. The cell is largely undifferentiated; only the nucleus (*N*) and a single electron-transparent vesicle (*ETV*) are seen in this section. No mitochondria are apparent. × 26,500.

cells grown anaerobically on the YE-G medium are strikingly undifferentiated; they contain little free cytoplasmic membrane, and they no longer have a large cell vacuole (Figs. 3 and 4). Examination of a large number of sections indicates that a vacuole is still formed, but is greatly diminished in size. Only the cell wall and plasma membrane, together with an occasional electron-transparent vesicle, are clearly evident (Fig. 3). Of considerable interest is the absence of readily recognizable mitochondria from these cells. Vague outlines are, indeed, seen in the cytoplasm of occasional cells grown anaerobically through a single growth cycle to the stationary phase (five to six cell divisions), and it may be that these outlines represent vestigial forms of the mitochondria (Fig. 4, arrow). However, these forms appear to be eliminated from cells cultured through several anaerobic growth cycles. Fig. 5 shows a typical cell which is the result of three anaerobic growth cycles and would correspond to an anaerobic cell over 14 divisions from the original inoculum. At this stage, the organism is characterized by the presence of a number of electron-opaque structures (one to four per section) of unknown nature. It is difficult to preserve adequately cells grown through several anaerobic growth cycles; the membranes of the cell are particularly poorly defined. The fragmentary nature of the nuclear envelope shown in Fig. 5 is typical. However, it should be emphasized that these cells are still viable and may be subcultured through several more anaerobic growth cycles.

CELLS GROWN ON THE YE-G + TWEEN 80 MEDIUM: The cytology of cells grown anaerobically on YE-G + Tween 80 is represented in

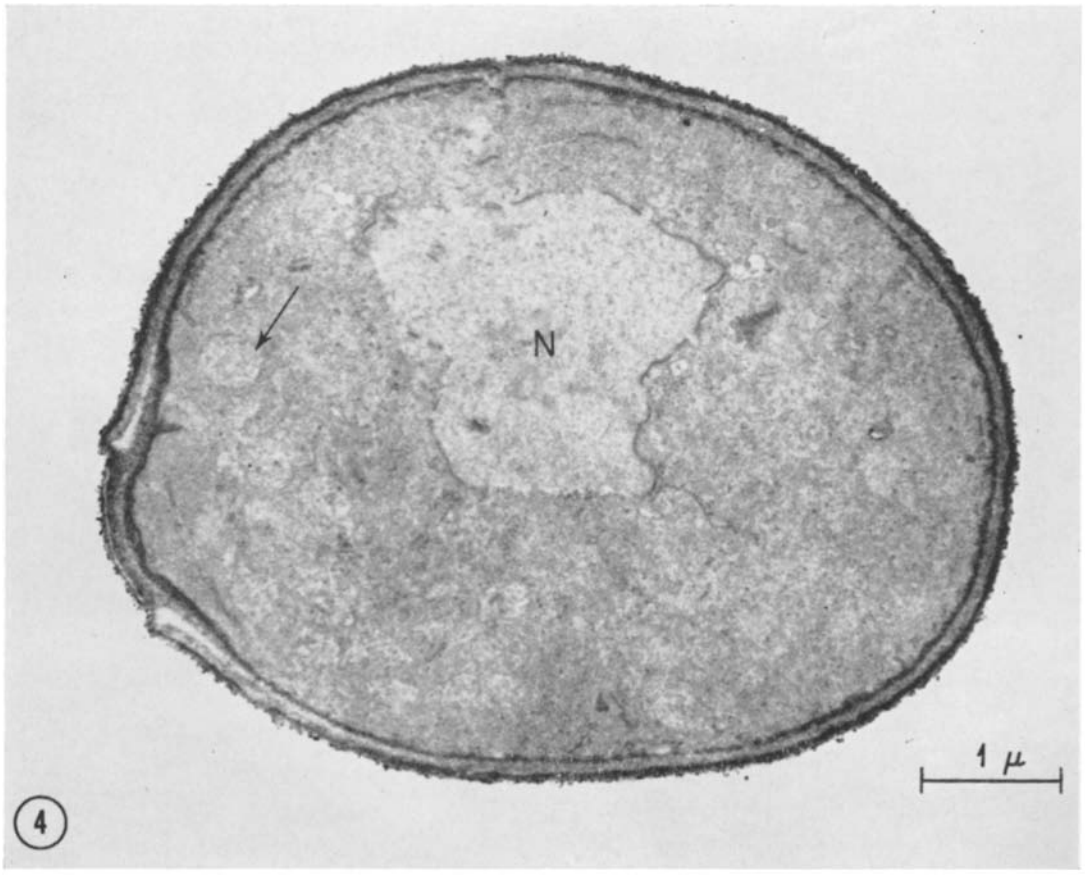

FIGURE 4 Section through a cell grown anaerobically on YE-G medium. The nuclear envelope is notably fragmentary in outline. The possible vestigial mitochondrial profile referred to in the text is indicated by the arrow. × 18,500.

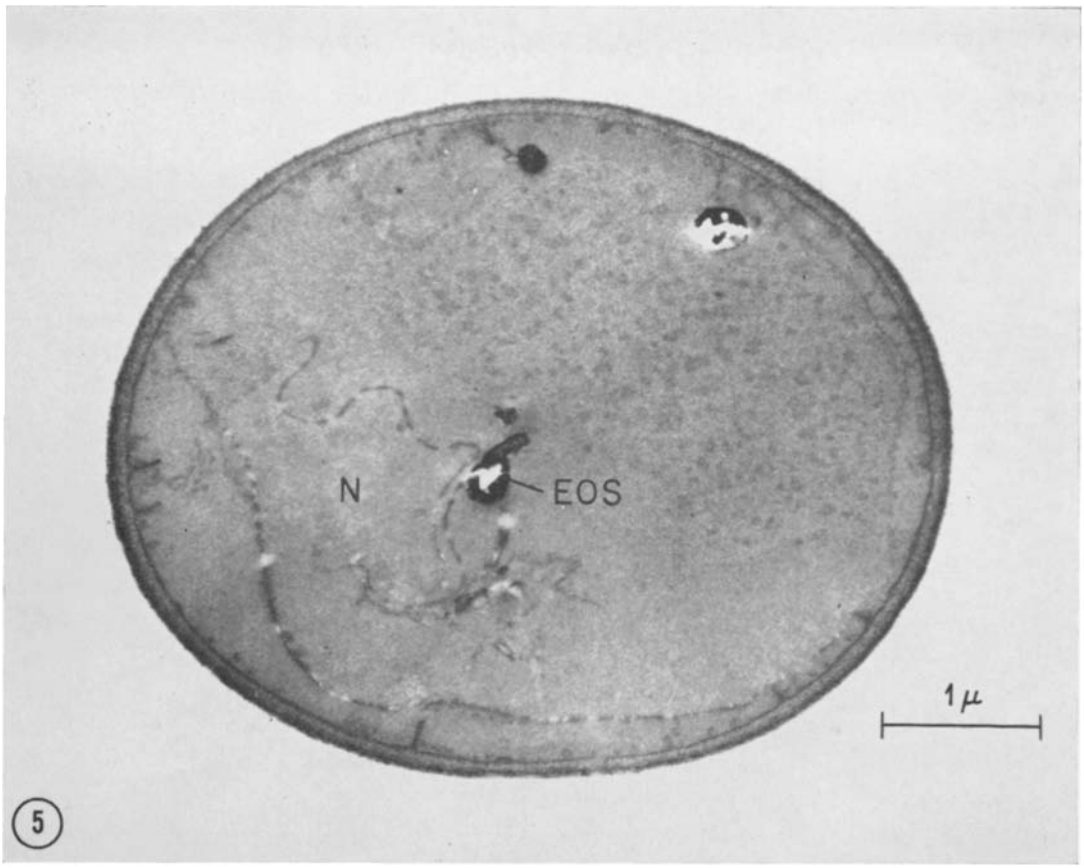

FIGURE 5 Cell resulting from three complete anaerobic growth cycles on YE-G medium. The plasma membrane is comparatively well preserved, but the nuclear envelope appears interrupted and there are signs of general fixation damage typical of these cells. The electron-opaque structures (*EOS*) are clearly seen. × 18,000.

Fig. 6. The organism contains large amounts of cytoplasmic membrane, and there are a number of electron-transparent areas which may have contained lipid. The membranes commonly assume two main forms: one is a laminar arrangement, made up of several unit membranes closely aligned and running through the cell for considerable distances; the other consists of small lengths of unit membrane seemingly scattered at random throughout the cytoplasm. These cells form a readily recognizable vacuole and nuclear envelope. Structures which might be interpreted as primitive mitochondrial profiles are occasionally seen in these cells (Fig. 6).

The characteristics described above are specifically associated with the availability of long-chain unsaturated fatty acids in the medium. Tween 80 may be replaced by either palmitoleic or oleic acid to produce similar results. On the other hand, neither palmitic nor stearic acid, free or in the form of Tween 40 or 60, respectively, supported the synthesis of the membranes. Indeed, the YE-G medium containing Tween 40 or 60 supported less growth than unsupplemented YE-G medium; the organism hydrolyzes the Tween to liberate the fatty acids, which form very obvious soap micelles. Presumably, high concentrations of free palmitic and stearic acids are inhibitory to anaerobic yeast cell growth.

CELLS GROWN ON THE YE-G + TWEEN 80 + ERGOSTEROL MEDIUM: The inclusion of ergosterol together with Tween 80 in the medium has profound effects on the cytology of the cell (Fig. 7); compared with the presence of

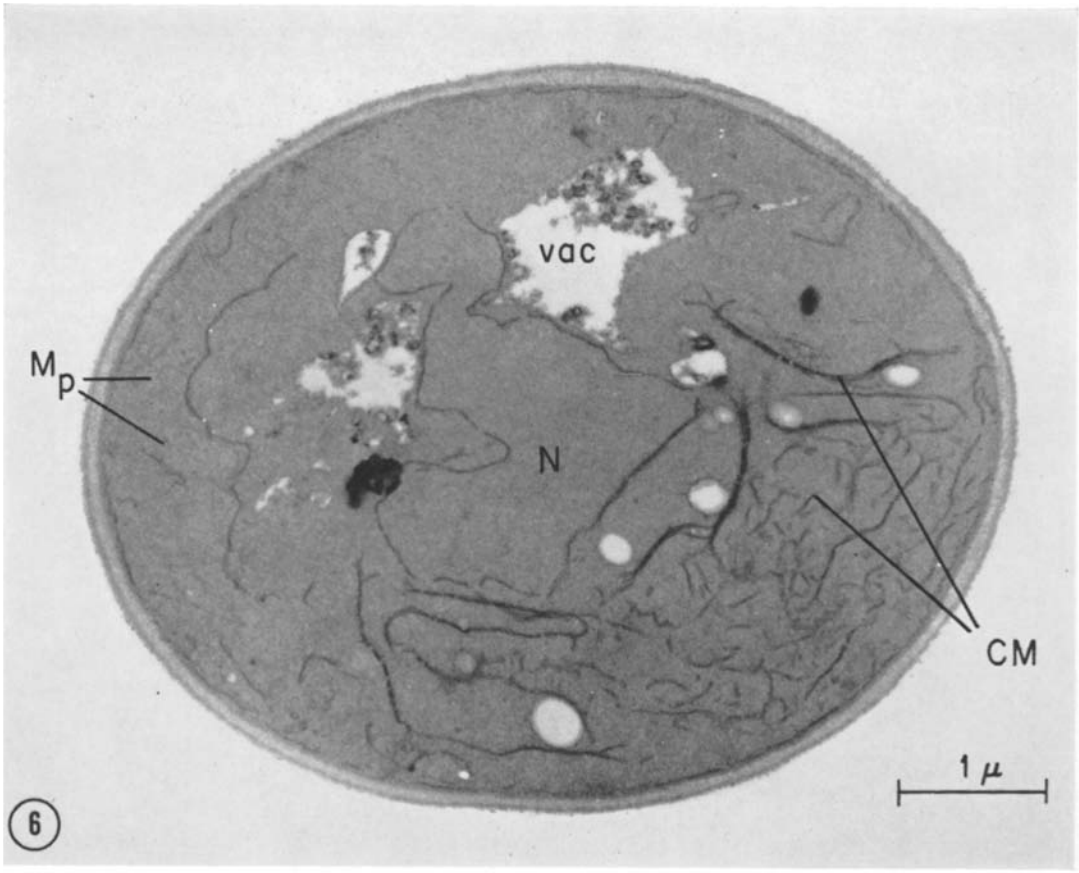

FIGURE 6 Cell grown anaerobically on YE-G + Tween 80 medium. The most characteristic feature of this cell type is the occurrence of considerable cytoplasmic membrane (*CM*). It is possible that primitive mitochondrial profiles (M_p) occur. × 6,500.

Tween 80 alone (Fig. 6), there is a big decrease in the cytoplasmic membrane. Ergosterol must diminish the synthesis of the cytoplasmic membrane or obviate the cell's need for such membrane. Infoldings of the plasma membrane and nuclear blebbing are sometimes observed in this cell. A small number of mitochondrial profiles are recognizable (Fig. 7). The membranes of these profiles are very poorly resolved compared with the other membranes in the cell, and also compared with the mitochondrial profiles of aerobically grown cells (Figs. 1 and 2).

CELLS GROWN ON THE YE-GAL + TWEEN 80 + ERGOSTEROL MEDIUM: Cells grown anaerobically on YE-GAL + Tween 80 + ergosterol medium contain clearly recognizable mitochondrial profiles (Fig. 8), better defined and in greater number than those in cells grown on the corresponding glucose-containing medium (Fig. 7). Otherwise, these two cell types appear to be identical. We interpret these observations as indicating that a glucose repression of mitochondrial profile synthesis occurs, even under anaerobic growth conditions. In further support of this view, it has been found that cells cultured on the YE-GAL medium without lipid supplements do contain occasional poorly defined mitochondrial profiles. These profiles are similar in definition to those seen in cells grown on YE-G medium plus lipid supplements (Fig. 7).

Absorption Compounds of Aerobic and Anaerobic Cells

There have been a number of studies on the absorption characteristics of anaerobically grown

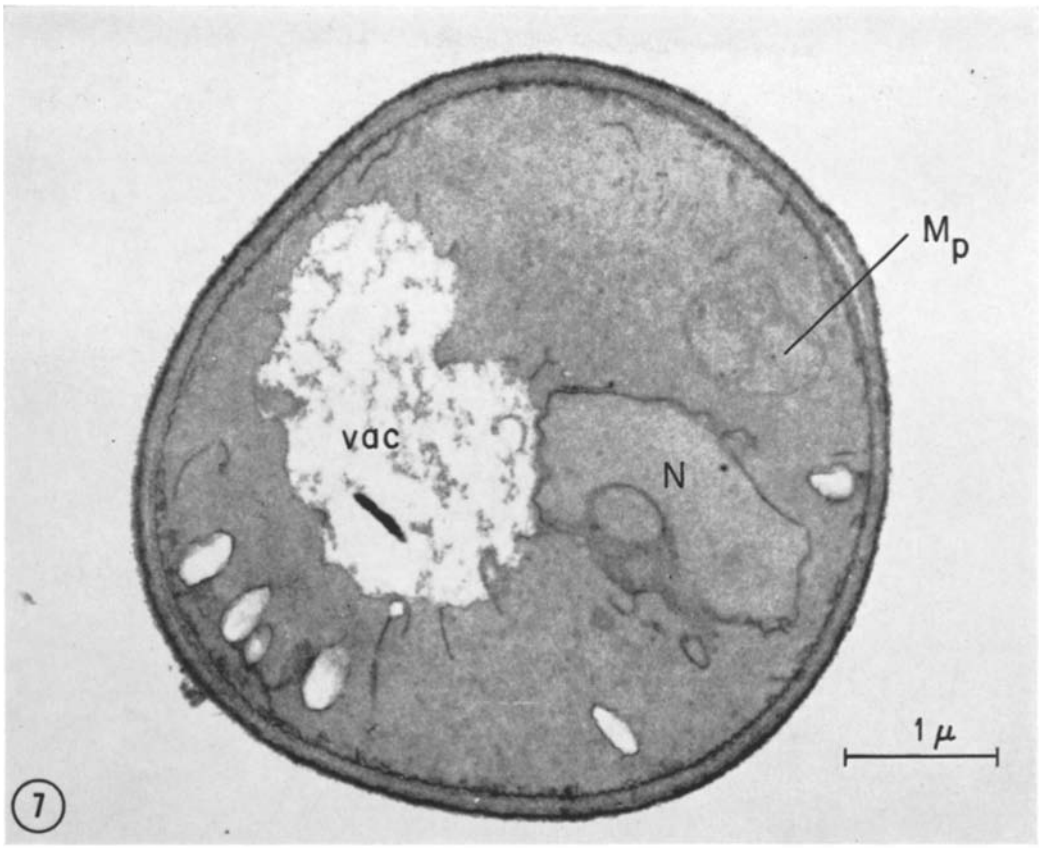

FIGURE 7 Cell grown anaerobically on YE-G + Tween 80 + ergosterol. A poorly resolved mitochondrial profile is apparent and is typical of these cells (M_p). × 20,500.

yeast, and at least seven different absorption bands, at 505, 540, 558, 575, 580, 590, and 632 mμ, have been reported (Lindenmayer and Estabrook, 1958; Chaix, 1961; Hennessy and Chance, 1964; Lindenmayer and Smith, 1964; Linnane, 1965). However, the nature, function, and significance of these compounds are uncertain, particularly as, at present, there is no clear rationale for their occurrence and not all authors report the presence of the complete range. Although our present studies shed no light on the nature and function of these compounds, it is pertinent to report that the absorption spectra of all the anaerobically grown cells differ from those of the aerobic cells, and, in addition, the absorption compounds present in the anaerobic cells vary, depending upon the composition of the growth media. These observations further emphasize the differences among the several anaerobic cell types.

The absorption spectra of the cell types depicted in the electron micrographs are shown in Fig. 9. The aerobically grown cells (curve *A*) have a classical cytochrome pattern; the absorption bands with maxima at 605, 562, and 551 mμ correspond to cytochromes a-a_3, b, and c-c_1. Cells grown anaerobically on the YE-G medium (curve *F*) appear to contain none of the aerobic cytochromes, but instead they show absorption bands around 632, 580, 555, 542, and 505 mμ. In cells grown on YE-G + Tween 80 + ergosterol (curve *D*), the 632-mμ band is no longer detectable and there are trace bands at 585 and 540 mμ. The major absorption bands in these cells are a well defined peak at 505 mμ and a smaller band with a peak at about 558 mμ. Further, the total amount of the absorbing compounds in these cells is much less than in cells grown on YE-G medium. Cells cultured on the YE-G medium with the sole addition of Tween 80

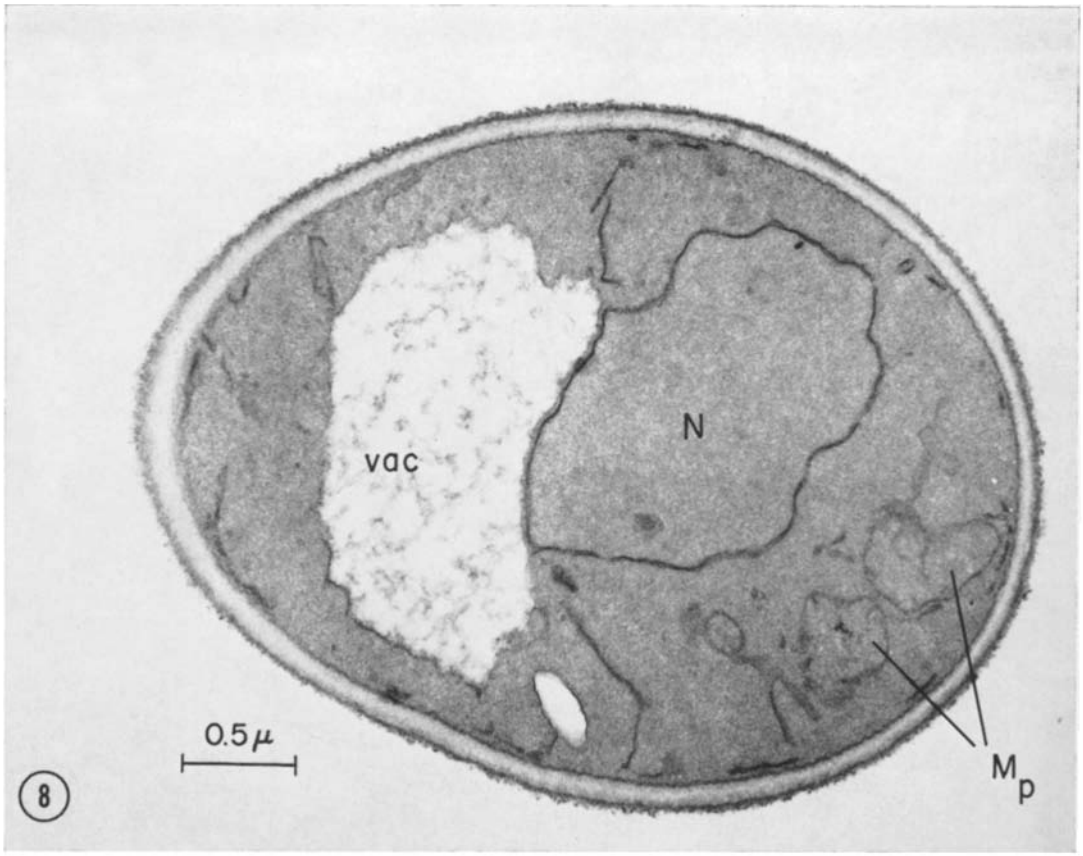

FIGURE 8 Cell grown anaerobically on YE-GAL + Tween 80 + ergosterol. The mitochondrial profiles of these cells are more clearly demarcated (M_p) than those in Fig. 7. × 26,500.

(curve *E*) have absorption characteristics intermediate between those of the other two types of glucose-grown cells.

Cells grown on YE-GAL (curve *B*) show five prominent absorption bands, at 632, 580, 557, 545, and 505 mμ. The total amount of pigment in these cells is noticeably higher than in any of the other anaerobic cell types. Cells grown on YE-GAL + Tween 80 + ergosterol (curve *C*) are characterized by the same absorption bands (585, 557, and 505 mμ) as are seen in the corresponding glucose-grown cells. They contain little or none of the 632- and 545-mμ compounds.

The spectral studies carried out at room temperature on the various cells indicate that probably five separate pigments with absorption bands at about 632, 580, 557, 542, and 505 mμ can be formed by anaerobically grown cells. Some of the absorption bands recorded at room temperature, for example those around 580 mμ, are very broad, and it is not possible to decide whether they represent single or fused peaks. The position can be clarified by examination of the absorption characteristics of the cells at the temperature of liquid nitrogen, a procedure which produces considerable narrowing of the bands and a very marked enhancement of the peak heights. The absorption maxima of the cell types observed at both room and liquid nitrogen temperatures are shown in Table I. It is confirmed that none of the aerobic cytochromes a-a_3, b, c_1, and c, which in liquid nitrogen show absorption maxima at 602, 559, 553, and 547 mμ, occurs in anaerobically grown cells. The broad 580-mμ absorption band observed at room temperature in most anaerobic cell types resolves into two bands with well defined maxima at about 583 and 575 mμ. However, these two bands do not appear to be due to a single compound, as they

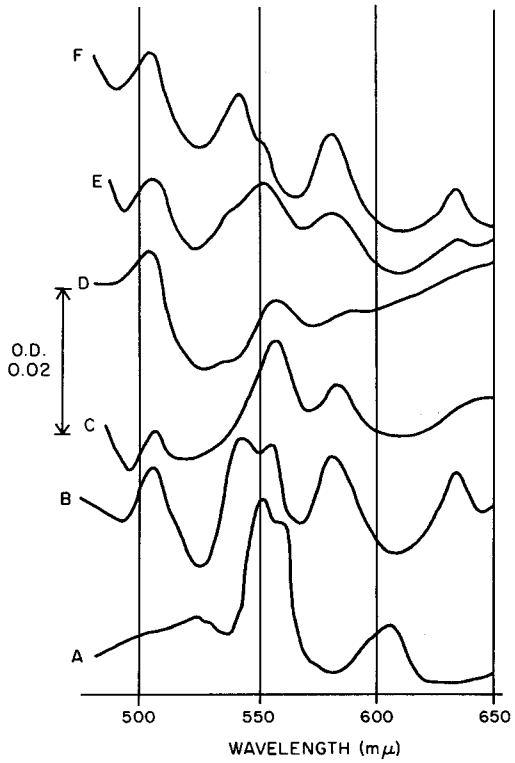

FIGURE 9 Direct room temperature spectra of whole yeast cells reduced with sodium dithionite. Curve *A* represents the spectrum of cells grown aerobically on YE-G medium. The other curves represent the spectra of cells grown anaerobically on the following media: *B*, YE-GAL; *C*, YE-GAL + Tween 80 + ergosterol; *D*, YE-G + Tween 80 + ergosterol; *E*, YE-G + Tween 80; *F*, YE-G. All spectra were recorded at cell densities of 25 mg dry weight per ml against a starch gel reference cuvette (Clark-Walker and Linnane, 1967).

are not always observed to accompany each other; thus the 583-mμ band has been seen to occur in cells grown on both the YE-GAL + Tween 80 + ergosterol medium and the YE-G + Tween 80 + ergosterol medium without observing a 575-mμ band; the converse has not been observed. The absorption band observed at about 557 mμ at room temperature, which is presumably due to cytochrome b_1, separates at low temperature into two peaks, at 557 and 551 mμ (Clark-Walker and Linnane, 1967). The absorption bands observed at room temperature at approximately 632, 545, and 505 mμ are not further resolved at the temperature of liquid nitrogen.

It is clear that, in addition to affecting the cytology of the cells, the composition of the medium also affects their pigment content. Apart from changes in the amounts of the compounds in the different cell types, it is of interest that the 632- and 540-mμ bands are most marked in the cells which are starved of ergosterol and unsaturated fatty acids and deficient in mitochondrial profiles. We have not detected cytochrome a-a_3 in any of the anaerobic cells, in confirmation of the results of Somlo and Fukahara (1965) and contrary to those of Tustanoff and Bartley (1964) who have described the occurrence of cytochrome *c* oxidase in cells grown anaerobically on galactose medium supplemented with Tween 80 and ergosterol.

DISCUSSION

It is clear from the results that not all the anaerobically grown yeast cells have the same properties. Thus, the cells may differ in their cytological characteristics, the nature and amount of their pigments, their enzymes (Lukins et al., 1966), and the nature and amount of their lipids (Jollow et al., 1968). The determinants of cell composition are the availability of oxygen, unsaturated fatty acids, and ergosterol, and a catabolite repression, most strongly exerted by glucose. The importance of the last factor in anaerobic cells is shown by the consistently greater development of mitochondrial profiles in galactose-containing media than in corresponding glucose-containing media.

Fixation of Yeast Cells

Osmium tetroxide has not proved to be a satisfactory fixative for vegetative yeast cells, as it does not appear to penetrate them (Vitols et al., 1961). Potassium permanganate is now generally used as fixative for such cells (Hashimoto et al., 1959; Conti and Naylor, 1960; Vitols et al., 1961; Yotsuyanagi, 1962 *a*; Hirano and Lindegren, 1963; Polakis et al., 1964; Wallace and Linnane, 1964), although it must be emphasized that their fine structure is still not so well defined and preserved as in animal and plant tissues. In addition, the present study has shown that the potassium permanganate fixation procedure must be varied as the composition of the media for anaerobic growth is changed. It has become apparent that the period of exposure of the cells to permanganate is critical and that the time taken for the achievement of adequate preservation is related to the degree of cytoplasmic differentiation of the cells which, in turn, is influenced by their lipid composition. It

TABLE I

Absorption Bands of Pigments of Various Yeast Cell Types Observed at Room Temperature and at the Temperature of Liquid Nitrogen

Growth medium	Absorption maxima (mμ)	
	Room temperature	Liquid nitrogen temperature
	Aerobic growth	
YE-G	605, 562, 550	602, 559, 553, 547
	Anaerobic growth	
YE-G	632, 580, 555, 542, 505	630, 583, 575, tr. 557* 551, 542, 505
YE-G + Tween 80	632, 580, 556, 540, 505	630, 583, 575, 557, 551, 542, 505
YE-G + Tween 80 + Ergosterol	tr. 585, 558, tr. 540, 505	583, 557, 551, 505
YE-GAL	632, 580, 557, 545, 505	630, 583, 575, 557, 551, 543, 505
YE-GAL + Tween 80 + Ergosterol	580, 557, 505	583, 557, 551, 505

The absorption maxima were obtained from direct spectra of whole cells grown to their stationary phase either aerobically or anaerobically on the indicated media. Details of the procedures are as described by Clark-Walker and Linnane (1967).

* Tr. (trace), indicates the presence of a small absorption band.

may well be that the various membranes of the cell constitute a framework which helps to maintain the cellular architecture throughout the fixation and embedding procedures. Again, the overall lipid composition of membranes has long been considered to be important in fixation by permanganate, and thus the difficulty of adequately preserving the cells grown anaerobically on YE-G medium could result from their unusual lipid composition (Jollow et al., 1968), rather than their undifferentiated nature. Whatever the reason, aerobic cells, with the most highly differentiated cytoplasm of the cell types studied, are sometimes adequately fixed by as little as 10 min of treatment with permanganate, while anaerobic cells grown on the YE-G medium require at least 4 hr. Even so, the YE-G anaerobic cells remain a difficult material for adequate fixation, and, on occasion after fixation, many of the cells are found extensively disrupted when examined in the electron microscope. The long period of fixation of the anaerobically grown cells evidently does not, in itself, produce artifacts, as the aerobically grown cells have a similar appearance whether fixed for 10 min or 4 hr.

Mitochondrial Membrane

Biochemical studies on the complex problem of the biogenesis of mitochondria have recently intensified, and a number of different experimental situations are being exploited. However, the totality of information currently available cannot be easily integrated into any generally acceptable hypothesis.

The main question posed by the present work is whether the cells grown anaerobically on the YE-G medium still contain a small number of mitochondrial profiles or whether these cells are completely free of the profiles. The data do not permit an unequivocal answer to this question, but the results do suggest the possibility that YE-G anaerobic cells may be devoid of mitochondrial profiles. However, it is clear that the number of mitochondrial profiles in cells grown anaerobically on different media varies, ranging from many per cell (YE-GAL + Tween 80 + ergosterol) to few or none (YE-G). It is well known that, on oxygenation of anaerobically grown cells, a proliferation of mitochondria is induced (Wallace and Linnane, 1964; Linnane, 1965); it follows that, were profiles absent from the YE-G anaerobic cells, then the mitochondrial profiles induced by oxygen would not arise from recognizable preexisting profiles. In the absence of more detailed knowledge, we have earlier referred to this process as de novo synthesis.

Evidence is accumulating that mitochondria have some degree of autonomy and that the

organelles may divide. The strength of this argument lies mostly in the recognition, in mitochondria, of DNA, RNA, ribosomes, a DNA-dependent RNA polymerase, and an amino acid–incorporating system with specific properties. However, perhaps the best evidence for self-duplication is still that provided by the experiments of Luck (1963 *a*, *b*) with a choline-requiring strain of *Neurospora crassa*. He has shown that, once incorporated into mitochondrial membranes, lecithin is conserved in these organelles as the cells divide and the mitochondrial numbers increase. However, this lecithin becomes distributed evenly in all the mitochondria, each of which must, therefore, have incorporated some newly synthesized lecithin from the cytoplasm. These results are consistent with the hypothesis that there is an accretion of cytoplasmic material into the organelles, which subsequently divide. Of interest in this context are cinemicrographic studies which clearly suggest that the organelles are continually undergoing fusions and divisions (Frederic, 1958).

Separation of mitochondrial inner and outer membranes by recently developed techniques has shown that their enzymes and lipid composition are very different (Green et al., 1966; Parsons et al., 1966; Sottocasa et al., 1967), and Sottocasa et al., (1967) have drawn attention to a similarity between the outer mitochondrial membrane and the smooth endoplasmic reticulum. These latter observations are consistent with the view that the outer membrane may arise from cytoplasmic elements. Furthermore, Linnane and colleagues have recently presented evidence that the protein-synthesizing capacity of yeast mitochondria appears to be restricted to the formation of only some components of the inner membrane while most of the protein components of the organelle are formed by a nonmitochondrial protein-synthesizing system, presumably the cytoplasmic ribosomes (Huang et al., 1966; Clark-Walker and Linnane, 1967; Linnane et al., 1967). If at least part of the inner membrane system as well as the outer membrane is made by a nonmitochondrial system, it will eventually be necessary to explain how inner membrane components are transferred through the outer membrane and integrated into the organelle during a process of mitochondrial growth and division.

Plasma and Nuclear Membranes

Cytologically, the simplest cell is that grown anaerobically on the YE-G medium. This cell is characterized by the presence of nuclear and plasma membranes, occasional electron-transparent vesicles, and the near absence of other membrane systems. As shown by Jollow et al. (1968), this growth medium is free of all but traces of sterol and unsaturated fatty acids, which are important structural components of the membranes of life forms more highly evolved than bacteria. The cells under these growth conditions contain only about 100 μg of ergosterol per gram dry weight of cells, and, of the total fatty acids, only about 2% are unsaturated (Jollow et al., 1968). However, as the cells will not grow on chemically defined media in the complete absence of these lipid components, these components are clearly essential to the survival of the organism, and traces of these compounds are presumably mandatory for the formation of functional nuclear and plasma membrane systems.

Vacuolar and Free Cytoplasmic Membrane

A large cell vacuole is formed by the anaerobic cells cultured on all media supplemented with unsaturated fatty acids.

The presence of large amounts of free cytoplasmic membrane in cells grown anaerobically on the YE-G + Tween 80 appears to be due to an imbalance in the available lipids. The membrane is formed only in the presence of an excess of unsaturated fatty acids, and the absence of ergosterol. Thus, cells grown on YE-G + Tween 80 + ergosterol, or YE-G + ergosterol, or YE-G media do not form the membrane.

The support of this investigation by the Australian National Health and Medical Research Council, and the United States Public Health Service (Grant GM 10496-05) is gratefully acknowledged.

Received for publication 28 September 1967.

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