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Formation and Degradation of Mitochondria in the Cell

I. Increasing Stability of Mitochondria during Aerobic Growth of *Saccharomyces cerevisiae*

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Summary

Prolonged deaeration of *Saccharomyces cerevisiae* cells results in degenerative changes in mitochondria which can be revealed when measuring the enzymic activities of the respiratory chain in isolated organelles and by electron microscope examination of the cells. The same changes are observed after a 3-h incubation of the cells with cyanide or carbonyl cyanide, m-chlorophenyl hydrazone in aerobic conditions. These results suggest the important role of oxidative phosphorylation in the maintenance of the integrity of mitochondria in the cell.

The sensitivity of yeast mitochondria to anaerobiosis and cyanide changes as the culture grows. Mitochondria are especially labile during the early exponential growth phase when their respiratory system and structure are not fully formed. Possible reasons for and the mechanism of degradation of mitochondria *in vivo* are discussed.

Introduction

One of the least known aspects of mitochondrial biogenesis is the principle of their stabilization in the cell. The question may be elucidated when studying the way in which various changes in the metabolism of the cell affect the structure and functions of mitochondria. Facultative anaerobic organisms make an ideal object for such investigations. Although the data so far obtained are not plentiful, it may be soundly postulated that mitochondria of facultative anaerobic

yeast are labile structures which degrade in certain conditions. This is supported by the data of Jayaraman *et al.* [1] who studied in detail the degradation of mitochondria of *Saccharomyces cerevisiae* under glucose repression. Meisel *et al.* [2] used the permanganate method of cell fixation to show that a prolonged incubation of some species of yeasts in anaerobic conditions results in the reduction of inner mitochodrial membranes. In our previous papers degradation and restoration of mitochondria on deaeration and subsequent aeration of aerobically grown *Saccharomyces cerevisiae* were described [3-5]. Simultaneously, Howell *et al.* [6] reported evidence showing that a prolonged deaeration of mycelia of a facultative anaerobic *Neurospora crassa* mutant causes reduction of the mitochondrial cristae. There are serious differences of opinion as to the level to which mitochondria may degrade in the yeast cell. Schatz *et al.* [7-9] believe that during anaerobiosis mitochondria degenerate to "promitochondria". In fact, the latter may be revealed even in anaerobically grown glutaraldehyde fixed cells [10] or those treated with the freeze-etching technique [9]. However, Howell *et al.* [6] reported that the number of distinguishable mitochondria markedly decreases after a long incubation of a facultatively anaerobic mutant of *Neurospora crassa.*

It has been shown in the present work that the degradation of the mitochondria of *Saccharomyces cerevisiae* may proceed to a lesser or greater extent, depending on the growth phase of the culture immediately preceding the deaeration of the medium. Mitochondria are especially labile at the early exponential phase, when their respiratory system and structure are not fully developed. In this case a 3-h anaerobiosis is most likely to cause a decrease in the average number of mitochondria per cell. Deaeration of yeast at the end of the exponential phase of aerobic growth induces some degenerative changes in mitochondria, their number in the cells being practically unchanged. There is a certain resemblance between the mitochondrial structures of these cells and cells during the early exponential phase of aerobic growth.

Materials and Methods

Organism and Growth Conditions. In all the experiments use was made of *Saccharomyces cerevisiae,* a wild-type diploid, strain No. 11 of the USSR National Collection of Yeast Cultures. The standard conditions of growth of the yeast, composition of the medium and the method of controlling the growth phase have been described elsewhere [3].

Thickening of Yeast Suspension. At certain growth phases the yeast were sedimented by centrifugation in cold (0- + 2°) and then the sediment

was diluted with the growth medium to a final concentration of 5% (wet weight). In all the experiments no noticeable cell division took place during long-term incubation. This procedure has been used by other authors [11]. Chloramphenicol (4 mg/ml) and cycloheximide (25 μg/ml), inhibitors of protein synthesis, did not affect either cell respiration or NADH oxidase and succinate oxidase activities of isolated mitochondria.

Isolation of Mitochondria. Mitochondria were isolated by the method of Tzagoloff [12], by disintegrating the cells in a homogenizer (14,000 rpm) after freezing them in liquid nitrogen. Mitochondria protein was determined as described by Lowry *et al.* [13].

Enzyme Assays. The NADH oxidase and succinate oxidase activities of the mitochondria were measured in the same conditions as used by Mackler *et al.* [14]. The succinate: ferricyanide reductase activity was measured in the conditions described by Rao *et al.* [15]. Cytochrome oxidase activity was measured according to Polakis *et al.* [16].

Electron Microscopy. The cells treated by lyophilized snail (*Helix pomatia*) gut juice were fixed with glutaraldehyde as described by Watson *et al.* [17]. The samples were sectioned in the LKB type-3 ultramicrotome. Microphotographs of the cell sections were made with an HU-11B (Hitachi) electron microscope.

Results

Incubation of Thickened Yeast Suspensions

All the experiments described below were carried out when cell division was suppressed (see "Materials and Methods"). Figure 1A shows the data on the growth of *Saccharomyces cerevisiae* cells and the development of cell respiration. One can see that 6 h of aerobic growth in standard conditions correspond to the early exponential phase when the cells consume oxygen at a low rate. After the thickening of the yeast, the respiratory system continues developing if the medium is aerated (Fig. 1B). During a 7-h incubation in such conditions the respiratory activity of the mitochondrial fraction achieves the level resembling that usually observed at the thirteenth hour of growth in standard conditions. This means that the thickening of yeast *per se* does not cause noticeable changes in the mitochondrial respiratory system.

Inactivation of the Mitochondrial Respiratory System Induced by Anaerobiosis and Cyanide

Mitochondria isolated from *Saccharomyces cerevisiae* cells after 6 h of aerobic growth are capable of oxidizing 20-30 nmoles of NADH per min

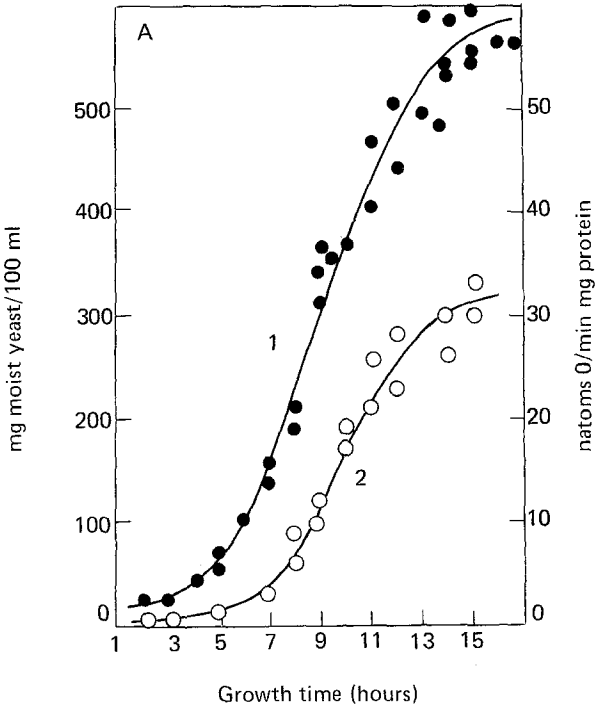

Figure 1A. Aerobic growth of *Saccharomyces cerevisiae* and development of cell respiration. Curve 1, concentration of yeast; curve 2, respiratory activity of the cells. Respiratory activity was measured polarographically at 30°. Assay medium contained 1% glucose in 50 mM potassium phosphate (pH 7.5).

per mg protein at 30°. This cyanide-insensitive activity does not decrease during incubation of the cells without oxygen (Fig. 1B).

After 7.5 h of aerobic growth mitochondria display a higher respiratory activity (70-80 nmoles of NADH per min per mg protein) which may be almost completely suppressed by cyanide. Incubation of these cells for 3 h in anaerobic conditions results in complete inactivation of the respiratory chain which reveals itself as a decrease in the NADH oxidase and succinate oxidase activities of mitochondria (Fig. 2A). In the same conditions the cytochrome oxidase and succinate: ferricyanide reductase activities change to a lesser degree.

As the respiratory system develops, its sensitivity to anaerobiosis *in vivo* decreases. When the yeast was deaerated after 13 h of aerobic growth (the end of the exponential phase, see Fig. 1A), the NADH oxidase and the succinate oxidase activities of the mitochondria

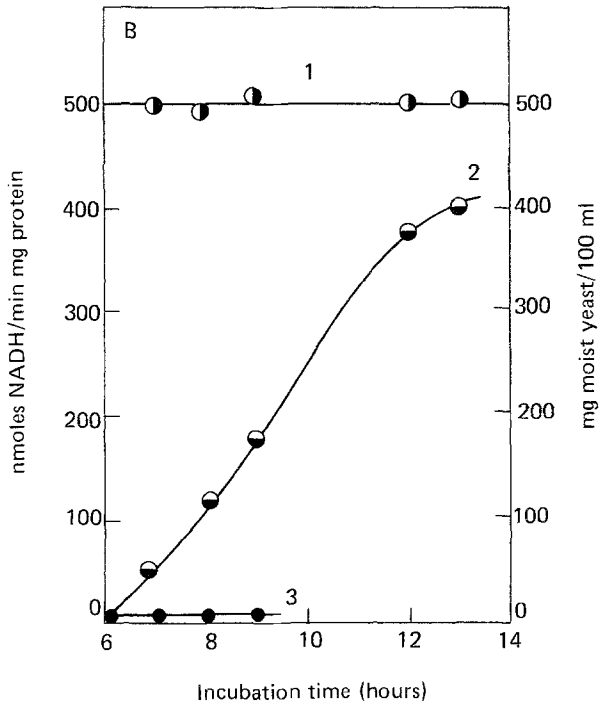

Figure 1B. Development of mitochondrial respiratory system during incubation of thickened yeast suspension. After 6 h of aerobic growth yeast was thickened and 5% cell suspension was incubated in aerobic (curve 2) or anaerobic (curve 3) conditions at 35°. In due course, aliquots of suspension were taken and mitochondrial fraction was isolated from yeast cells to measure NADH oxidase activity. Curve 1, concentration of yeast suspension during the experiment.

diminished much more slowly than those of 7.5-h yeast. In fact, after a 3-h anaerobiosis these activities decreased by 50% only (Fig. 2B).

A comparison of the results for 7.5-h and 13-h yeasts shows that the changes in mitochondria described above are not associated with starvation of the cells during incubation. Indeed, had starvation been the cause of inactivation of the respiratory system, the resulting effect would have been much more pronounced in the case of the 13-h yeast.

It is noteworthy that the incubation of yeast with cyanide in aerobic conditions results in the same effects as the incubation without cyanide in the absence of oxygen (Fig. 3).

As will be demonstrated below, the most probable reason for the NADH oxidase and succinate oxidase activities to go down is degenerative changes in the structure of mitochondrial membranes. The

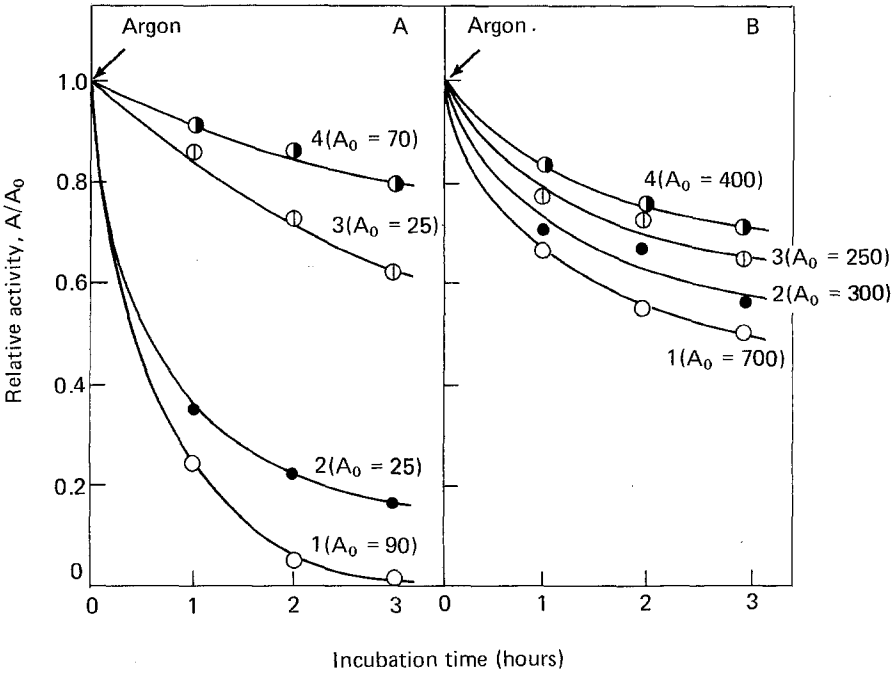

Figure 2. Degradation of mitochondrial respiratory system under deaeration of *Saccharomyces cerevisiae* yeast cells at the early (A) and late (B) exponential growth phase. Yeast was grown aerobically at 35°. In due course aliquots of yeast suspension were taken, mitochondrial fraction was isolated and the following activities were measured: 1, NADH oxidase; 2, succinate oxidase; 3, cytochrome *c* oxidase and 4, succinate dehydrogenase. In this figure activities are expressed as nmoles of NADH, succinate and ferrocytochrome *c* oxidized per min per mg protein at 30°.

character of these changes is such that the activities of the respiratory chain components (for example, succinate dehydrogenase and cytochrome *c* oxidase) decrease insignificantly, whereas the electron transfer is interrupted almost completely in the system as a whole (i.e. from NADH or succinate to oxygen) (see Fig. 2A).

The Effect of Chloramphenicol and Cycloheximide on the Development of the Mitochondrial Respiratory System of Yeast

Figure 3 shows that chloramphenicol and cycloheximide added to the yeast suspension after 7.5 h of aerobic growth suppress the development of the respiratory system of mitochondria. However, these protein synthesis inhibitors do not induce degradation of the respiratory system

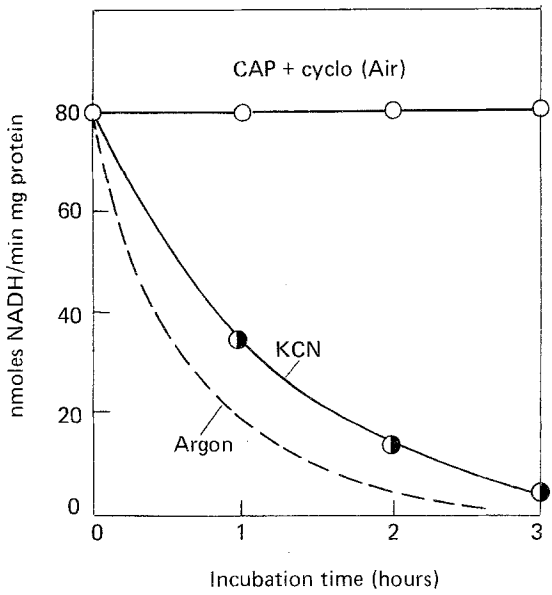

Figure 3. The effects of protein synthesis inhibitors and cyanide on development of mitochondrial respiratory system during incubation of thickened yeast suspension. Yeast was grown aerobically for 7.5 h. Then cell suspension was thickened and incubated aerobically in the presence of chloramphenicol, CAP, (4 mg/ml) and cycloheximide, CYCLO, (25 μg/ml) or in the presence of KCN (3 mM). Other conditions were the same as indicated above.

during the 3-h incubation of thickened yeast suspension. These results allow the conclusion to be made that inactivation of the respiratory system in anaerobic conditions is not likely to be associated with the cessation of the synthesis of mitochondrial proteins.

Our results agree with the evidence reported by some authors [18] and do not agree with the results of other authors [19, 20], which may, apparently, be explained by the differences in experimental conditions (objects of investigation, period to be observed, moment of addition of inhibitors to the incubation medium, etc.).

Morphological Changes in Mitochondria Induced by Anaerobiosis at Different Growth Phases

The data of electron microscopy confirm the suggestion that mitochondria are especially sensitive to anaerobiosis at early stages of development and that their stability markedly increases at the end of the exponential growth phase when the development of the respiratory system nears completion. Figure 5 summarizes the data for yeast

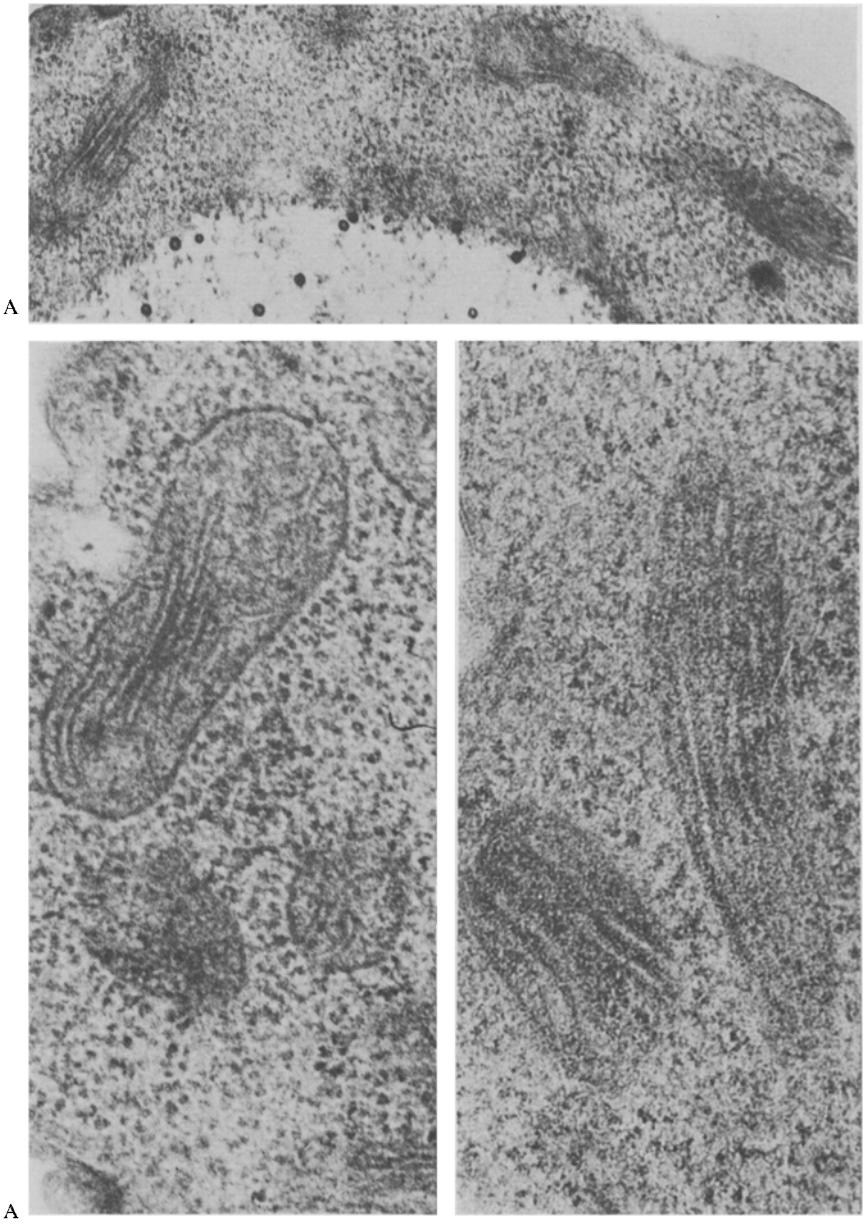

Figure 4. *Saccharomyces cerevisiae* yeast cells grown aerobically for 13 h. Mitochondria of non-deaerated yeast cells (A), x97,000 and (A), x127,000, and those of the cells deaerated for 30 min (B), x150,000. In all micrographs mitochondria are in orthodox conformation.

deaerated after 13 h of aerobic growth. Almost all the mitochondria in such cells before deaeration possess the orthodox conformation, i.e. that with long narrow cristae and a matrix occupying most of the mitochondrial volume (Fig. 4A, A′). A 30-min incubation of yeast in the absence of oxygen does not entail any noticeable changes in the structure of mitochondria (Fig. 4B). After a 3-h anaerobiosis various types of mitochondrial structures may be observed in the cells. Among them, particles in condensed (or intermediate) conformation (Fig. 5A) and promitochondria-like particles (Fig. 5B) occur most frequently. The former are characterized by numerous irregularly shaped intracristal spaces and a condensed matrix.* As for particles of the second type, they have a dense matrix and a small number of electron transparent spaces which never form the interconnected network. Such a morphology suggests that the inner membranes in the particles of the second type have a primitive organization. Watson *et al.* [17] have previously described promitochondria of *Saccharomyces cerevisiae* cells grown anaerobically in the presence of Tween-80 and ergosterol in a similar way. The appearance of the promitochondria-like particles may be considered as being a result of the reduction of cristae during prolonged deaeration of yeast cells.

In addition to these particles, after 3-h anaerobiosis there appear mitochondrial structures with still more distinct signs of degeneration. These are irregularly shaped enlarged mitochondria which in some cases adhere to cytoplasmic vesicles of unknown nature (Fig. 5C). Other types of degenerate mitochondria are particles devoid (or almost devoid) of the granular matrix but containing broken cristae (Fig. 5D); or those having a dense matrix but absolutely indistinguishable cristae (Fig. 5E). According to Hackenbrock [22], similar degenerative changes in mitochondria may be observed after incubation in 0.25 M sucrose at 30°.

In the cells subjected to a 3-h anaerobiosis one may also observe regularly shaped electron transparent regions which do not seem to have the limiting membrane (Fig. 5F).

Carbonyl cyanide, m-chlorophenyl hydrazone† added to the medium after 13 h of aerobic growth of yeast caused just the same morphological changes in their mitochondria. Incubation of yeast with carbonyl cyanide, m-chlorophenyl hydrazone for 3 h resulted in the appearance of promitochondria-like particles (Fig. 6B), mitochondria with a diluted matrix, particles with a dense matrix occupying most of their volume, enlarged mitochondria, free membranes, etc. As in the case of

* According to King *et al.* (see ref. 21), mitochondria in condensed conformation appear in the HeLa and L cells after a long incubation with chloramphenicol or ethidium bromide. This morphological change typical of the last stage of degradation of mitochondria is believed to be a result of disturbed respiratory functions.

† A two-fold increase in cell respiration was observed immediately on addition of carbonyl cyanide, m-chlorophenyl hydrazone (10^{-6}M).

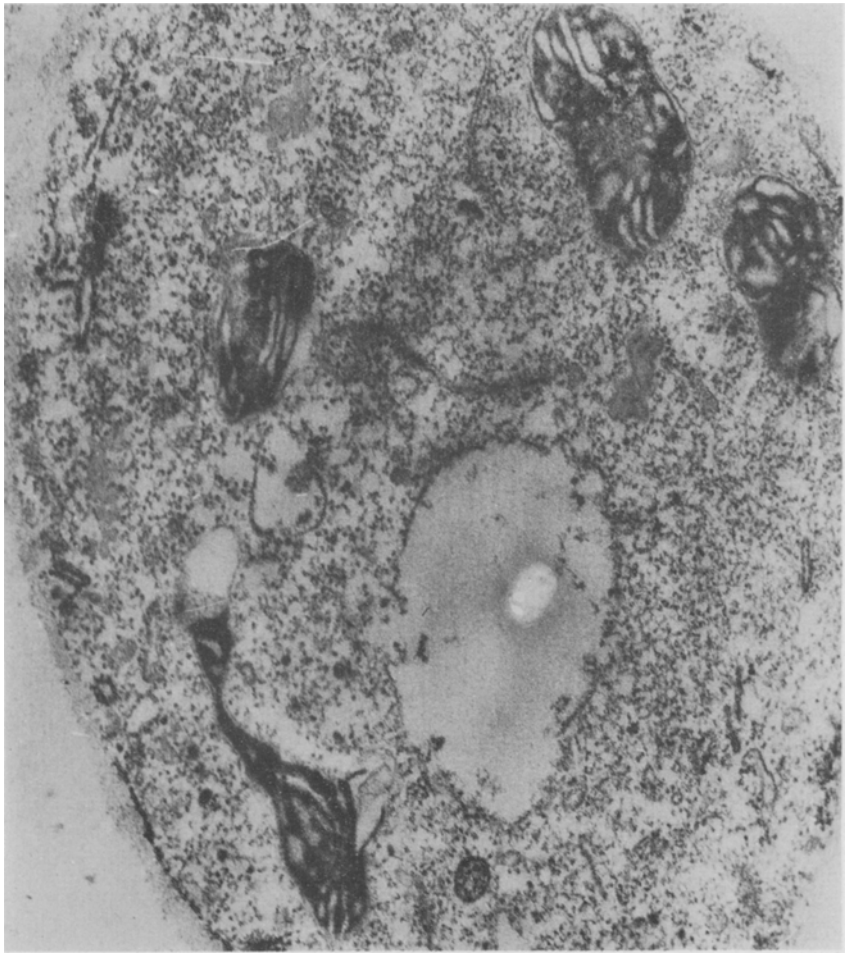

Fig. 5A

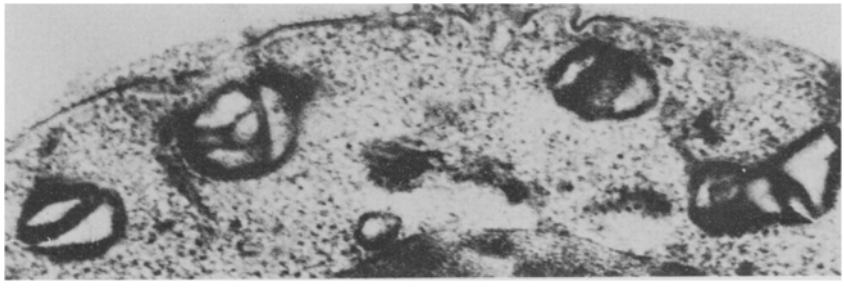

Fig. 5B

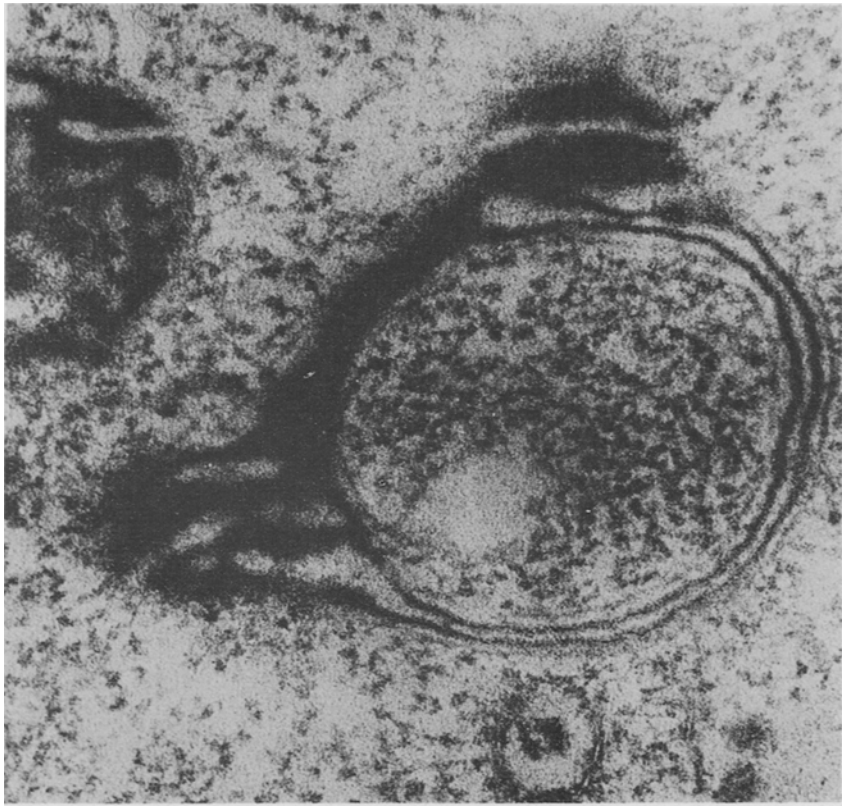

Fig. 5C

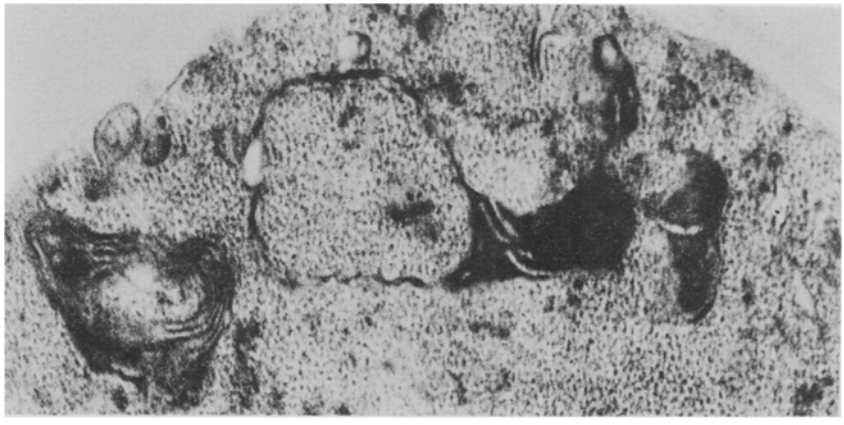

Fig. 5C

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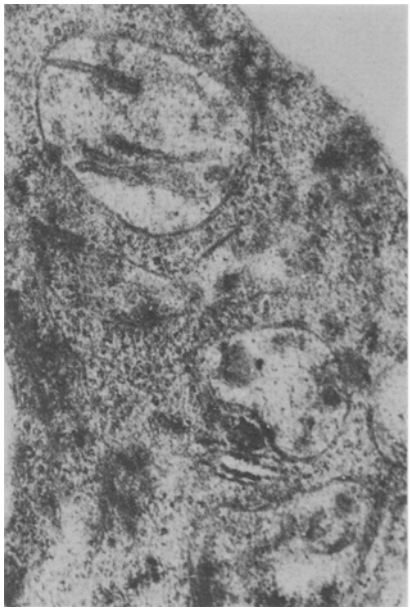


Fig. 5D

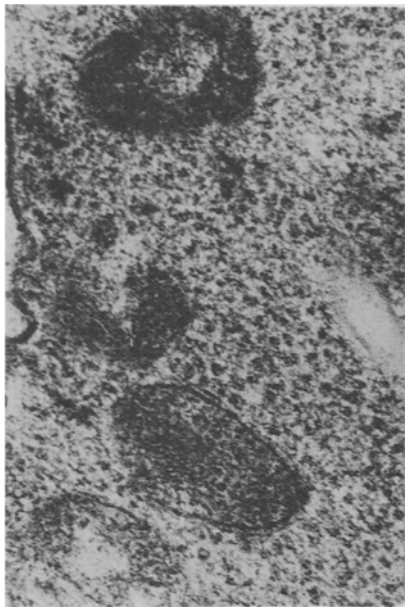


Fig. 5E

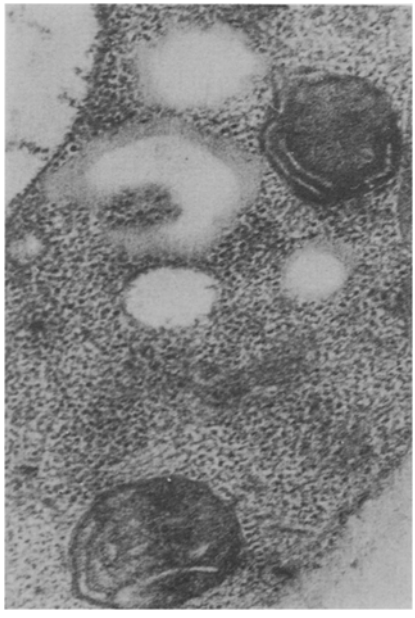


Fig. 5F

anaerobiosis, these degenerate structures were not observed after a half-hour incubation of the yeast with the uncoupler (Fig. 6A) and hence were not an immediate result of a change in the functional state of the mitochondria.

The effect of anaerobiosis on mitochondria was still more pronounced when the yeast were deaerated after 7.5 h of aerobic growth. At this stage the respiratory system of yeast was yet poorly developed (the intensity of cell respiration was only 15-20% of that at the end of the exponential phase). Figure 7 shows that an extreme heterogeneity of subcellular particles is characteristic for 7.5-h cells. Among such particles all types of degenerate mitochondria occur which are commonly observed after prolonged anaerobiosis of 13-h cells or their incubation with an uncoupler in aerobic conditions (see Figs 5 and 6). Special attention should be paid to the abundance of the particles with a dense matrix and with a few electron transparent oval or round spaces. Watson *et al.* [17] qualified such particles as promitochondria of *Saccharomyces cerevisiae* yeast grown anaerobically without Tween-80 and ergosterol.

After a 3-h deaeration of 7.5-h cells no new types of particles different from those described above are observed. However, in this case the average number of mitochondrial structures per cell section is reduced by approximately 50%. This phenomenon can not be attributed to separate organelles being fused together, because the appearance of enlarged mitochondria was not characteristic of deaerated 7.5-h yeast cells. The deaerated cells are peculiar in that they contain more vacuoles, and in some of the latter membranous structures can be discerned (Figs 8A, B). Deaeration of 13-h cells under the same conditions results in serious degenerative changes in the mitochondria (Fig. 5), but the average number of mitochondrial structures per cell section remains the same (see Table).

Discussion

The present paper describes different types of degenerate mitochondrial structures which appear on deaeration of facultative anaerobic yeast *Saccharomyces cerevisiae* at various phases of aerobic growth. The

Figure 5. Morphological changes in mitochondria of deaerated *Saccharomyces cerevisiae* yeast cells. Culture was grown for 13 h (the end of the exponential phase) and then deaerated for 3 h. A, mitochondria in the condensed conformation, x55,000; B, mitochondrial structures resembling promitochondria of anaerobically grown yeast, x68,000; C, C', irregularly shaped enlarged mitochondria ("monster mitochondria"), x44,000 and x150,000; D, particles with a diluted matrix containing broken membranes, x61,000; E, particles with a double limiting membrane, a granular matrix and without distinguishable cristae, x98,000; F, electron transparent visicles and particles with a few narrow cristae and a dense matrix occupying most of the particle volume, x69,000.

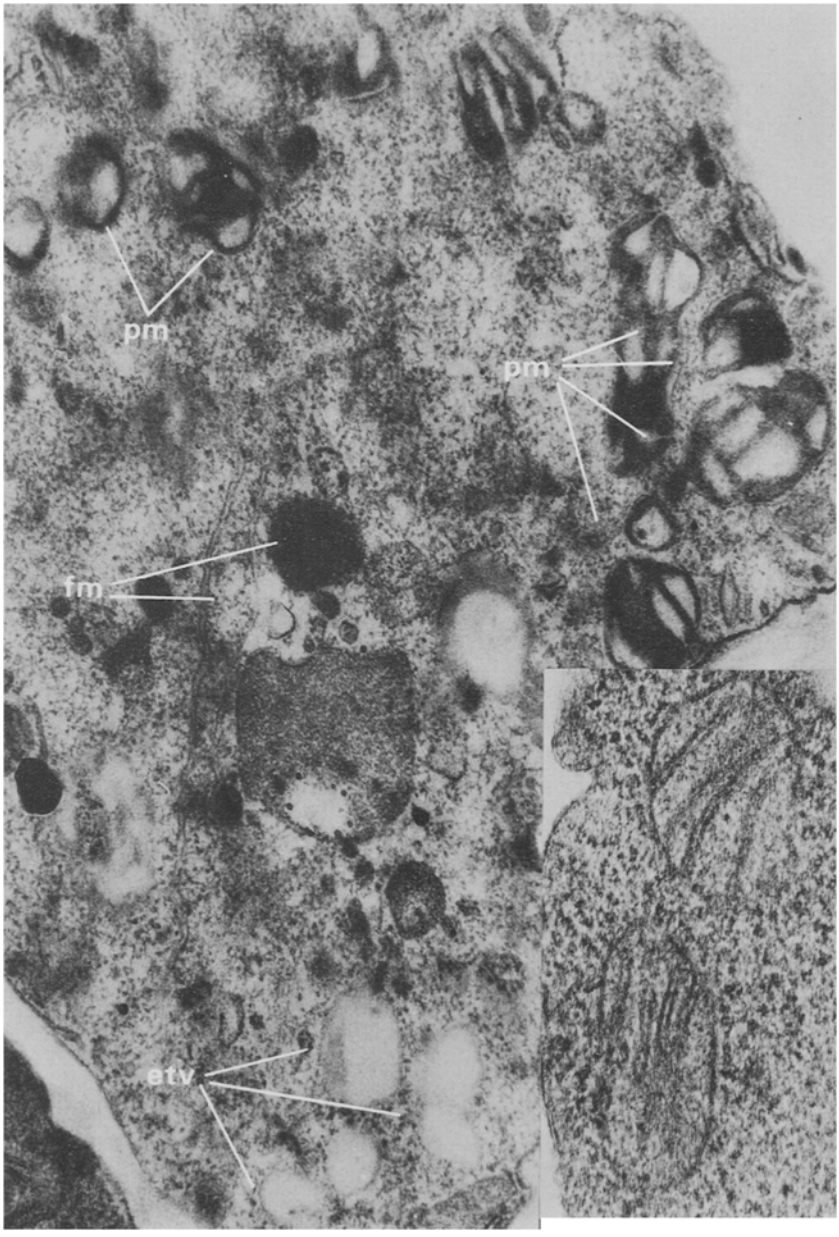


Fig. 6B

Fig. 6A

TABLE I. Effect of 3-h deaeration of *Saccharomyces cerevisiae* suspension on the average number of mitochondria per cell section

Number of experiment	Average number of mitochondria (and mitochondria-like particles)			
	7.5 h of aerobic growth		13 h of aerobic growth	
	Before anaerobiosis	After 3 h of anaerobiosis	Before anaerobiosis	After 3 h of anaerobiosis
1	2.5 (60)	1.2 (60)	5 (50)	5 (50)
2	2.7 (60)	1.1 (60)	6 (50)	6 (50)

Figures in brackets indicate the number of cells examined.

morphological investigations are combined with measurements of the respiratory activity of the mitochondrial fraction. The main conclusion of this work is that anaerobiosis causes the most profound changes in mitochondria at the early stages of their formation.

Prolonged deaeration of yeast at the end of the exponential growth phase which is characterized by fully developed mitochondria results in the reduction of cristae and appearance of particles resembling promitochondria of anaerobically grown *Saccharomyces cerevisiae* [17]. This type of morphological changes in mitochondria is not the only one. In the present work other cytoplasmic structures are described which may be looked upon as degenerate mitochondria.

During the early exponential phase yeast cells have a characteristically high number of promitochondria-like particles. A strictly definite heterogeneity of the mitochondrial structures is another morphological peculiarity of such cells. Unlike the 13-h cells, in which anaerobiosis induces degeneration of mitochondria without decreasing their number, young cells lose about half of their mitochondrial structures for 3 h of anaerobiosis, as follows from the electron microscope evidence. Howell *et al.* [6] have recently described a similar phenomenon in a facultative anaerobic *Neurospora crassa* mutant subjected to prolonged deaeration. The authors did not overlook the fact that this could be an artifact arising in the course of fixation. But in accordance with Watson *et al.*

Figure 6. Morphological changes in the mitochondria of *Saccharomyces cerevisiae* yeast cells incubated with carbonyl cyanide,m-chlorophenyl hydrazone. Culture was grown aerobically for 13 h (the end of the exponential phase), after which the medium was supplemented with carbonyl cyanide,m-chlorophenyl hydrazone (1×10^{-6}M). A, mitochondria (orthodox conformation) of yeast cells incubated for 30 min with the uncoupler, x100,000; B, yeast cells incubated with the uncoupler for 3 h: promitochondria-like particles (pm), free membranes (fm) and electron transparent vesicles (etv), x45,000.

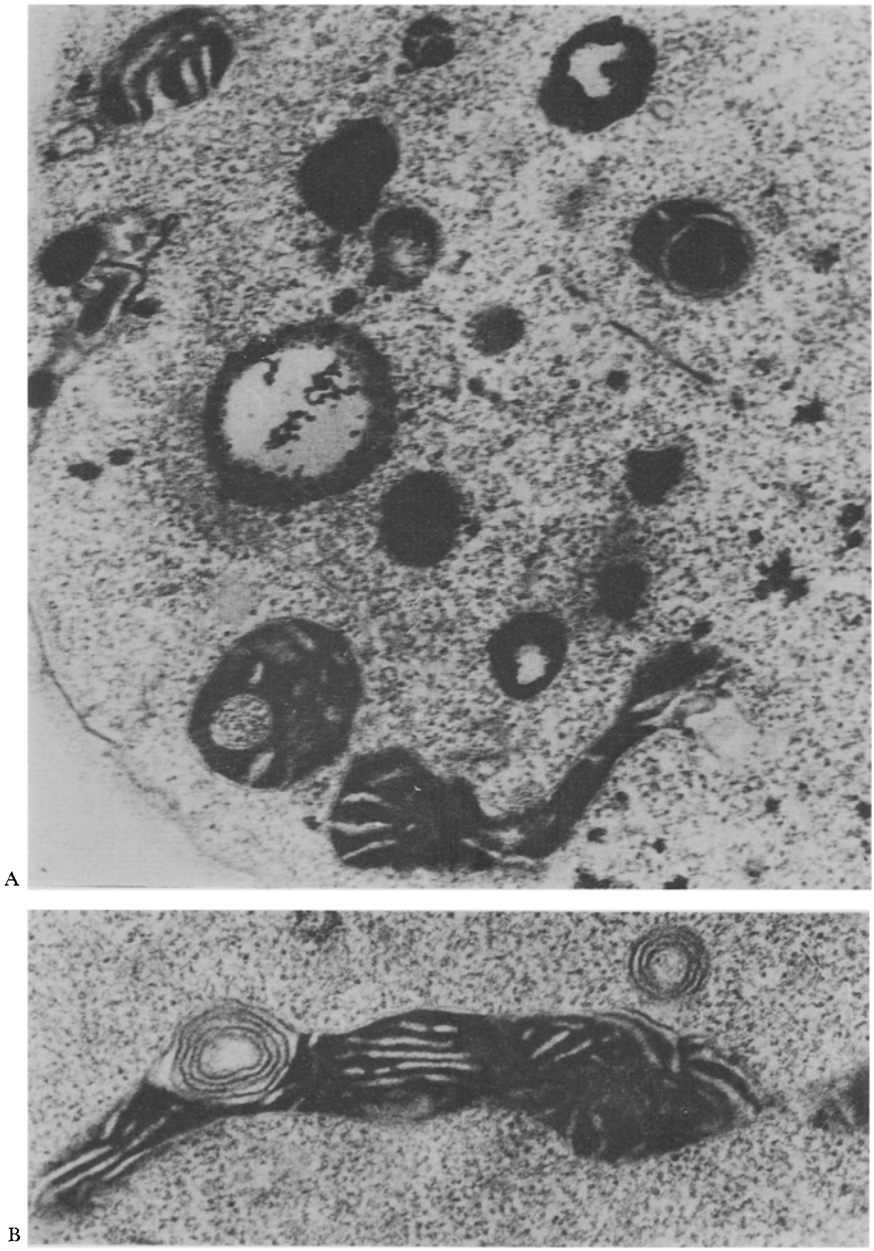
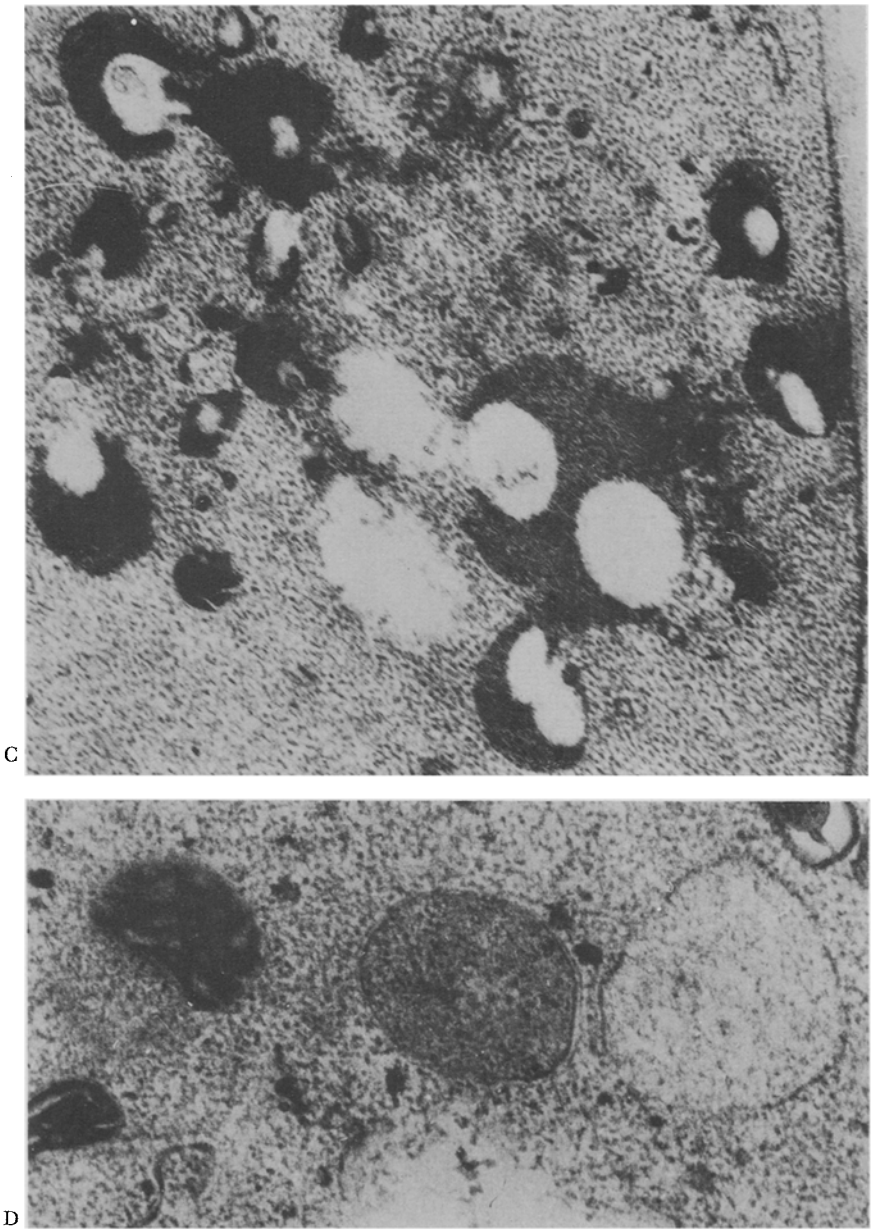


Figure 7. *Saccharomyces cerevisiae* yeast cells grown aerobically for 7.5 h (early exponential phase). A, various types of mitochondrial structures, ×72,000; B, "giant"



C

D

mitochondria, x67,000; C, promitochondria-like particles, x57,000; D, particle with a granular matrix and a double limiting membrane (of Fig. 5E), x80,000.

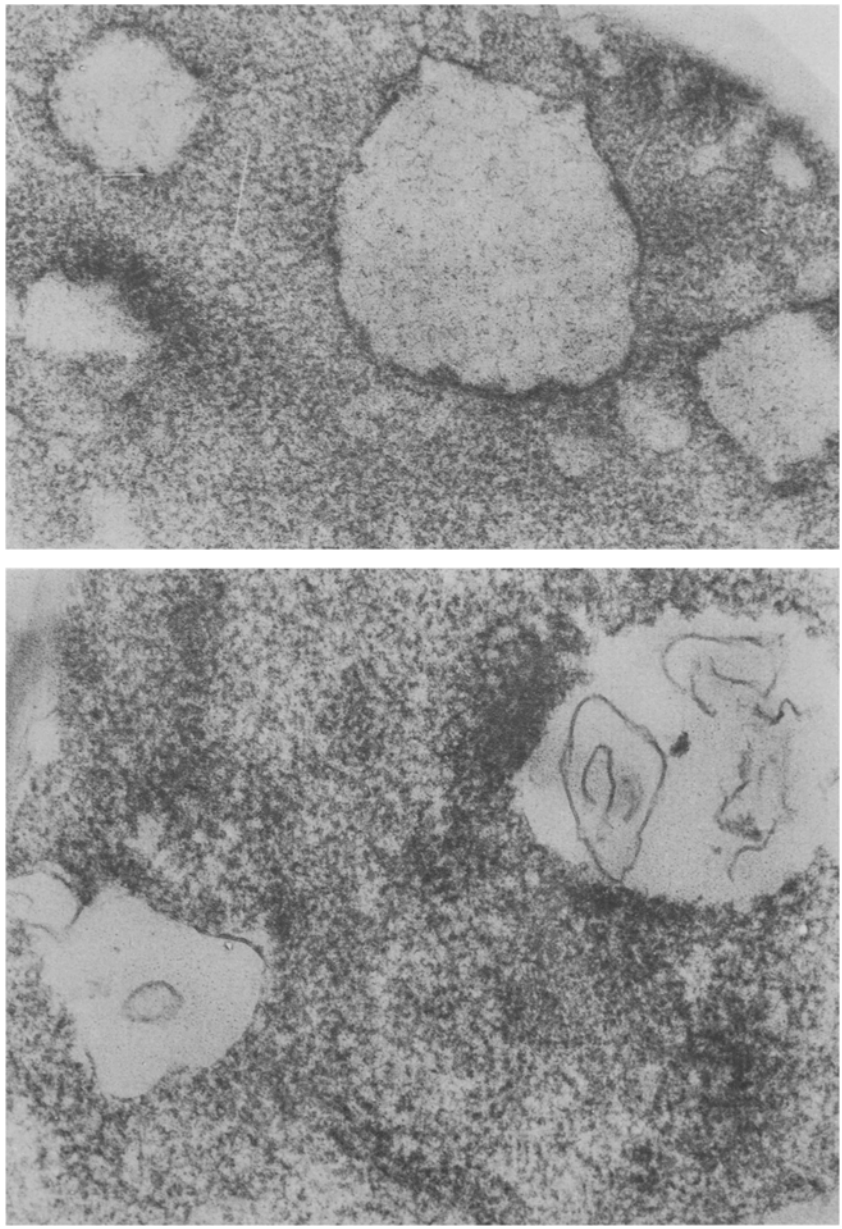


Figure 8. Morphological changes in the cytoplasm of the yeast cells grown aerobically for 7.5 h (early exponential phase) and deaerated for 3 h. A, vacuolization of the cytoplasm, x57,000; B, membraneous structures in vacuoles, x66,000.

[17] and also Damsky *et al.* [10], glutaraldehyde fixation allows mitochondrial structures to be revealed even in the conditions most unfavourable for mitochondria formation (anaerobic growth in a glucose medium containing no unsaturated fatty acids and ergosterol). Therefore, it is possible to believe that the decrease in the number of mitochondrial structures of yeast cells revealed by electron microscopy really testifies to the mitochondria dissipating in certain conditions. It is hardly possible to say now what is the final result of dissipation.

Dharmalingam and Jayaraman in their two short communications [23, 24] suggest that the disintegration of mitochondrial membranes in conditions of glucose repression is due to the action of cytoplasmic phospholipases. These authors found that glucose repression induces activation of phospholipases and derepression is accompanied by a decrease in their activity. Nothing is known so far about where yeast phospholipases are localized and how their activity is controlled.

One of the conclusions of this work is that a correlation may be found between the extent to which mitochondria degrade and vacuolization of the yeast cells. It has been suggested that the cell vacuole of yeast is a single large lysosome [25, 26]. It is appropriate to recall here the former electron microscope data by Ashford and Porter [27], Novikoff and Essner [28] and also the electron micrographs presented in the review of de Duve and Wattiaux [29] which illustrate digestion of mitochondria by lysosomes in animal cells. The idea of the participation of lysosomes in digestion of mitochondria was convincingly supported in the recent work of Travis and Travis [30] who studied the cytological changes appearing in miocardic cells with age. Of course, this is a formal analogy but in any case the suggestion of the existence of a correlation between the degradation of mitochondria and the increase in the number of vacuoles in yeast cells deserves further study.

In this connection the recent work of King *et al.* [21] should be mentioned. These authors found, when studying chloramphenicol-induced morphological changes in HeLa and L cells, that a considerable number of vacuoles and cisterns appear at the late stages of degradation of mitochondria.

Finally, a few words about the reason for the degradation of mitochondria on deaeration of aerobically grown *Saccharomyces cerevisiae*. It has been shown in our previous work [31] that anaerobiosis, respiratory inhibitors and uncouplers induce inactivation of the respiratory chain of mitochondria and in all cases this process proceeds at one and the same rate. In this work the resemblance of the effects caused by anaerobiosis and an uncoupler, carbonyl cyanide, m-chlorophenyl hydrazone, has been confirmed by morphological investigations. These data suggest that oxidative phosphorylation is necessary for the integrity of mitochondria to be maintained *in vivo* and that any interruption of oxidative phosphorylation results in degradation

of mitochondria. The mechanism of this phenomenon is unknown. One of the working hypotheses being developed in our laboratory is that the oxidative phosphorylation system (and consequently the whole inner mitochondrial membrane), when in the functioning state, takes on a specific conformation which is resistant to heating and to the action of lytic enzymes (proteinases and phospholipase A) [32-35]. According to another hypothesis, the role of oxidative phosphorylation in stabilizing mitochondria may be that of maintaining a sufficiently high level of intramitochondrial ATP which is necessary for free fatty acids to be reincorporated into mitochondrial phospholipids* [36, 37]. Apparently, arrest of biosynthesis of mitochondrial proteins is not among the factors responsible for degradation of mitochondria (see Fig. 3).

De Duve and Wattiaux [29], when discussing the possible role of lysosomes in the "cell economy", suggested that one of the functions of these organelles is to digest "ballast material". The present and the previous works carried out in this laboratory have prompted us to the conclusion that mitochondria which for this or that reason prove incapable of effectively carrying out oxidative phosphorylation may be such "ballast material". This point of view may prove beneficial in studying the principles of the formation of mitochondria.

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* Free fatty acids, as is known [38-42], accumulate in mitochondria under the action of mitochondrial or lysosomal phospholipase A and are likely to be responsible for swelling and degradation of mitochondria.

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