MITOCHONDRIAL BIOGENESIS IN *NEUROSPORA CRASSA*

I. An Ultrastructural and Biochemical Investigation of the Effects of Anaerobiosis and Chloramphenicol Inhibition

NEIL HOWELL, CAROL A. ZUICHES, and KENNETH D. MUNKRES

From the Laboratories of Molecular Biology and Genetics, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT

The isolation of a new class of mutants permitting facultative anaerobiosis in *Neurospora crassa* is described. Backcross analyses to the obligate aerobe prototroph (An^-) indicate single nuclear gene inheritance (An^-/An^+). An^+ and An^- are indistinguishable in morphology and growth rates under aerobic conditions. Anaerobic growth requires nutritional supplements that are dispensable for aerobic growth. Conidiogenesis, conidial germination, and vegetative growth rate are suppressed by anaerobiosis. An^+ mutants produce substantial quantities of ethanol under anaerobic conditions. Anaerobiosis and chloramphenicol both affect mitochondrial enzyme activity and morphology. Chloramphenicol inhibition leads to reduction in cytochrome oxidase and swollen mitochondria with few cristae. Anaerobiosis leads to reduction in both cytochrome oxidase and malate dehydrogenase activities, enlarged mitochondria with fewer cristae, enlarged nuclei, and other alterations in cellular morphology. The fine structure of anaerobically grown cells changes with the time of anaerobic growth. We conclude that either inhibition of mitochondrial membrane synthesis or inhibition of respiration might lead to the observed alterations in mitochondria.

INTRODUCTION

Facultative anaerobiosis, the capacity to grow and reproduce in either aerobic or anaerobic conditions, has arisen sporadically during the evolution of fungi, particularly among the yeasts and some animal pathogens. The genetic and biochemical bases of the differences between facultative anaerobes and obligate aerobes have received little attention, although such information could be of economic and medical value and could contribute to the understanding of cellular morphogenesis. For example, Warburg's pioneering investigations suggest that cancerous cells, in contrast to normal cells, derive the bulk of their energy by fermentation rather than oxidative metabolism (1). There is also evidence implicating a causal relationship between oxygen metabolism and dimorphism, a fungal morphogenetic phenomenon (2). This relationship may involve reversible changes in the mechanisms of cellular division.

Among yeasts, facultative anaerobiosis and fermentation appear to approach an all-or-none effect (3). For example, two closely related species may differentially exhibit strict aerobiosis and facultative anaerobiosis. These findings suggested

that such facultative behavior may be determined by a limited number of genes; such a possibility led us to examine 12 wild-type strains of *Neurospora crassa* from various geographical areas of the world in an attempt to detect growth under partially anaerobic conditions. Preliminary experiments in our laboratory, supported by previous studies (6–8), revealed that none of these strains was capable of anaerobic growth. Our studies included a Javanese strain, since Went (4) noted that a Javanese strain could ferment and grow anaerobically; this particular strain had been used in the preparation of a fermented food and presumably had undergone some selection for its fermentative properties (5).

The failure to detect natural variants allowing anaerobic growth led to the induction of mutants with facultative capacity. This report describes the isolation of such facultative anaerobe mutants and the characterization of some of their biological properties. A mutant of this type allows analysis of the effects of reduced oxygen tension on mitochondrial morphology, biogenesis, and biochemistry as well as cellular ultrastructure.

The present studies provide additional insights from analysis of the effects of a specific genetic alteration on cell growth and organelle biogenesis. Furthermore, the ability to grow under anaerobic conditions should enhance the survival of respiration-deficient mutants. The collection of numerous respiration-deficient mutants, both nuclear and extrachromosomal, will aid greatly in the elucidation of the genetic bases of mitochondrial biogenesis. The present studies are concerned with the use of the An^{+} strain for other biochemical and genetic studies rather than a detailed comparison with the An^{-} wild-type.

MATERIALS AND METHODS

Cultures and Media

LIQUID CULTURES: Two kinds of culture media are employed to enforce aerobic or anaerobic growth. For aerobic nonfermentive growth, standard Fries' minimal medium (9) supplemented with either 1% glucose or sucrose is used. For anaerobic growth, an enriched medium employed for anaerobic growth of *Saccharomyces cerevisiae* is used (10). This medium (YECE) differs primarily from minimal medium in that it contains yeast extract and casamino acids. In addition, Tween 80 (Atlas Chemical Industries, Inc., Wilmington, Del.) (5 g/liter) (a source of unsaturated fatty acids) and ergosterol (20 mg/ml) are generally added to YECE since they stimulate anaerobic growth of *Saccharomyces* (10, 11). To reduce contamination from spore-forming bacteria, double concentration (2×) YECE (without glucose) is autoclaved 15 min at 120°C, allowed to stand overnight, and autoclaved a second time. Glucose (2×, 4–20%) in distilled water is separately autoclaved and added in equal volume to the final YECE after cooling. Stock solutions are stored at 4°C.

Media containing chloramphenicol are prepared by adding the correct amount of dry antibiotic to partially cooled flasks of autoclaved media.

AGAR SLANTS AND PLATES: Fries' minimal, glucose agar slants, and sorbose-sucrose plates are prepared by conventional technique; colonial growth on plates is induced by adding sorbose (0.8%) and sucrose (0.1%) (12). Colonial growth on enriched agar medium is done with YESSCE. In this medium, in place of glucose, a hot solution of sorbose (1.6%) and sucrose (0.2%) is added, just after autoclaving, in equal volumes to hot, autoclaved YECE containing 3% agar.

ANAEROBIC CULTURES: Anaerobic cultures in liquid YEGCE are grown in three ways. *Method No. 1:* YEGCE (300 ml) is added to a 50 ml vacuum filtration flask fitted with a fritted glass gas dispersion tube, two outlets, and a magnetic stir bar. The gas dispersion tube is connected to a tank of oil-pumped nitrogen. Air is withdrawn from the system with an aspirator. Nitrogen is bubbled through the culture medium and water trap for at least 30 min. The conidial inoculum is added to the flask before the initial evacuation. Incubation is done at 30°C with stirring. *Method No. 2*: Cultures are incubated in an anerobic oven (National Appliance Company, Portland, Ore.) at 30°C after three cycles of evacuation to a vacuum of −74 cm Hg and flushing with nitrogen to atmospheric pressure. Oxygen concentration in the medium, determined with a portable oxygen electrode, is generally 0.2% or less. *Method No. 3*: Cultures are incubated in an atmosphere of H_2/CO_2 in a Brewer-Allgeier "Gas-Pack" jar (13) (Fisher Scientific Company, Pittsburgh, Pa.) according to the directions of the manufacturer.

Isolation of Facultative Anaerobic Mutants

The procedure is diagrammed in Fig. 1. Conidia of wild-type 74A (FGSC No. 936) were harvested from slants after 4–5 days of growth and filtered through glass wool. 1×10^{10} conidia were irradiated with an ultraviolet germicidal lamp (General Electric G3078) to 10% survival and incubated anaerobically by method No. 1 for 5–7 days. Traces of mycelial growth were removed by filtration through cheesecloth. The filter was washed several times with sterile fresh medium to remove entrapped conidia. The cheesecloth and mycelia were transferred to a

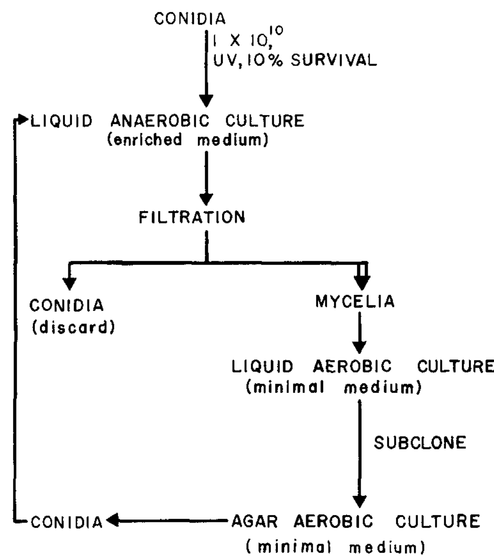


FIGURE 1 Flow sheet of the procedure of isolation of facultative anaerobic mutants of *Neurospora*.

flask of Fries' minimal medium and incubated for several days at 30°C on a shaker. Mycelial fragments were subcloned to Fries' minimal slants and allowed to become conidial. Three additional cycles of anaerobic-aerobic growth were carried out. Six subclones were then plated on YEGCE, and single "yeastlike" colony isolates were made after incubation for 7 days in an anerobic jar (method No. 3). These isolates, after aerobic conidiation on YEGCE slants, were tested in 1-ml cultures of YEGCE for growth in the anaerobic jar. Additional selection was made on the basis of these tests.

Genetic Analyses

Each of six mutant sublines was backcrossed to the obligate aerobe wild-type (3.1a, FGSC No. 935) on 2% corn meal agar (0.1% sucrose) slants at 25°C. Random ascospores were collected 1 month after crossing, heat shocked (60°C, 60 min), plated on YESSCE, and incubated aerobically for 4 days at 30°C. Single colonies were transferred to YEGCE slants for conidia of progeny isolates, incubated anaerobically by method No. 3 for 7 days at 30°C, and scored visually for growth during that period. Progeny exhibiting positive responses were scored as An^{+}. An^{-} progeny and the original parent exhibited no growth after 1 month's incubation under these conditions.

An^{+} backcross progeny were tested for the mating type locus (*A/a*) by the method of Perkins (14). Other unselected phenotypes observed were glycerol utilization (Gly^{+}/Gly^{-}) and chloramphenicol sensitivity (CAP^{R}/CAP^{S}). It is likely that these unselected phenotypes arose in the original wild-types since similar differences can be observed in crosses of two An^{-} strains. Aerobic growth on glycerol was tested in 3-ml tubes containing 1 ml of YEGLYCE (YECE + 1% glycerol). Inhibition of aerobic growth by chloramphenicol was tested in YEGLYCE + CAP (4 mg/ml).

Biochemical Methods

Ethanol concentrations in culture filtrates were determined by an enzymatic method (Sigma Chemical Co., St. Louis, Mo.; method No. 330). Other conditions are described in the legend of Table II.

Methods of preparing cellular extracts and mitochondria, assays for malate dehydrogenase, and protein determination were previously described (15). Cytochrome oxidase activity was determined spectrophotometrically with dithionite-reduced cytochrome *c* by the method of Sherman and Slonimski (16). All enzyme assays were performed at 33°C with a Gilford 2000 recording spectrophotometer (Gilford Instrument Company, Oberlin, Ohio).

Electron Microscopy

Conidia were inoculated centrally onto a dialysis membrane layered on a Petri plate containing YEGCE supplemented with 1% glucose and 1% agar. The dialysis membrane was sterilized in 70% ethanol and washed in sterile distilled water before overlaying the membrane onto the medium. The plates were incubated aerobically at 30°C for 20–24 hr or anaerobically for 3 days at 30°C by method No. 3. Mycelial pads on the dialysis membrane were lifted from Petri plates with tweezers and transferred to 5 ml of freshly prepared fixative in a Petri plate. Fixation was allowed to proceed for 1 hr (at room temperature) in a solution (pH 7.3) of sodium cacodylate (0.05 M), sucrose (0.065 M), glutaraldehyde (0.33 M), and acrolein (0.54 M). The osmolarity of the fixation medium was 0.70 M, the iso-osmolar concentration of *Neurospora* mycelia (17).

After fixation, mycelia and dialysis membrane were soaked overnight in a freshly prepared solution of sodium cacodylate (0.1 M, pH 7.3) and sucrose (0.12 M). Hyphal tips were removed in segments about 2–5 mm from the periphery of the mycelial pad and postfixed at 0°C for 1 hr in a freshly prepared solution containing osmium tetroxide (1%), sodium cacodylate (0.1 M), and 0.125 M sucrose. Subsequently, the hyphal tips were washed several times in distilled water, stained in 0.5% aqueous uranyl acetate for 30 min, and washed twice with distilled water. Hyphal tips were dehydrated by successive incubations in 50, 70, 95, and 100% ethanol with two 5-min intervals in each solution, followed by two 10-min intervals in

100% propylene oxide. Stepwise infiltration with Epon-Araldite (1:0.8) and DMP-30 catalyst (1%) in propylene oxide mixtures of 50, 70, and 90% plastic were made at 24-hr intervals with a final 4 hr period in 100% Epon-Araldite. Plastic was cured by a 2 day incubation at 40°C followed by 5 days at 60°C. Thin sections were cut with a glass knife and an LKB II microtome and stained by the method of Frasca and Parks (18) with uranyl magnesium acetate and lead citrate. Sections were examined in a Hitachi HU-11-C electron microscope at an accelerating voltage of 75 kv.

Chemicals

Antimycin A, D-chloramphenicol, cytochrome *c* (Type VI), ergosterol, and Tween 80 were obtained from Sigma Chemical Co., St. Louis, Mo. Acrolein, glutaraldehyde, and propylene oxide were products of Eastman Organic Chemicals, Rochester, N.Y. Sodium cacodylate and uranyl magnesium acetate were obtained from K & K Laboratories, Inc., Plainview, N.Y. Other chemicals and their sources were: osmium tetroxide (Fisher Scientific Co., Pittsburgh, Pa.), uranyl acetate (Baker Chemical Co., Phillipsburg, N.J.), Epon (Shell Chemical Co., New York), Araldite (Ciba Products Co., Summit, N.J.), DMP-30 (Rohm and Haas Co., Philadelphia, Pa.), and sucrose (enzyme grade) (Mann Research Labs Inc., New York).

Glutaraldehyde was purified by passage through a column of Dowex-2 (Dow Chemical Co., Midland, Mich.) ion exchange resin in ammonium form.

RESULTS

Genetic Analyses

Six subclones of facultative anaerobe mutants were isolated after initial UV irradiation and three cycles of enrichment for growth as illustrated in Fig. 1 and detailed in Materials and Methods. Because of the experimental design, one cannot ascertain whether the mutants were induced or of spontaneous origin, or whether they were of independent origin.

Backcross analyses clearly indicate a 1:1 segregation of the phenotype, suggesting a single nuclear gene (Table I). No infertility or ascospore inviability was observed. Facultative anaerobiosis is designated as An^+ without any implications of dominance or recessiveness. Some degree of heterogeneity of growth rate of various An^+ progeny from single crosses was observed. Possibly, additional modifier genes were segregating. Other nonselected phenotypes, glycerol utilization (Gly^+) and chloramphenicol resistance (CAP^R), as well as the mating type locus (*A*) also segregated 1:1 without any evident linkage to one another or An^+. In this context, we note that deliberate selection in *S. cerevisiae* yielded Gly^- and CAP^R mutants inherited as nuclear genes (19, 20).

For most subsequent studies, two lines were selected from An^+-2 backcross progeny: An^+ 2-B-12-3*a*, CAP^S, Gly^+ and An^+ 2-B-16-3*A*, CAP^S, Gly^+.

General Observations of An^+ Mutants

An^+ and An^- strains are morphologically indistinguishable and grow at equal rates in aerobic conditions on either enriched or minimal medium.

In *S. cerevisiae*, two metabolites whose synthesis requires molecular oxygen, ergosterol, and unsaturated fatty acids (Tween 80) are required for optimal anaerobic growth (10, 11). Anaerobic growth of An^+ strains is apparently more nutritionally fastidious, as in yeasts, since Fries' minimal glucose medium, alone or with Tween 80 and ergosterol supplement, does not support growth. Only enriched medium (YEGCE) supports

TABLE I
Segregation of Facultative Anaerobe Locus (An^+) in Backcross to Wild-Type (An^-)

	Number of progeny genotypes					
	An^+ Exp. No.			An^- Exp. No.		
Cross*	1	2	3	1	2	3
An^+ 2 × 3.1a (An^-)	11	10	8	9	10	12
An^+ 5 × 3.1a (An^-)	8	10	9	12	10	11
An^+ 6 × 3.1a (An^-)	9			11		
An^+ 10 × 3.1a (An^-)	8			7		

* The parent on the left was protoperithecial. An^+ progeny were capable of anaerobic growth from conidial inocula (method No. 3) in 2–5 days whereas no growth of An^- occurs in at least 1 month.

anaerobic growth of An^+ and addition of Tween 80 and ergosterol to YEGCE does not improve the growth rate. Linnane and associates reported (10) that yeast extract alone serves as a limited source of these nutrients for anaerobic growth of *S. cerevisiae*. In the present study, the components of the rich media which allow growth under anaerobic conditions have not been determined.

Anaerobic growth rates and total cell yield are less than in aerobic culture. Under partial anaerobiosis in an anaerobic oven, in which the oxygen tension is about 1% that of air, on liquid YEGCE at 30°C, the apparent doubling time is at least 8 hr in contrast to 2 hr in maximally aerated cultures.

TABLE II

Ethanol Accumulation by Anaerobic Cultures of Facultative Anaerobe Mutants of Neurospora crassa

A. *Complete Anaerobiosis*

Strain	Ethanol
	% w/v
An^+-2-B-12-3a	0.535
An^+-6-B-10a	0.525
An^+-6-B-20a	0.427
An^+-5-B-8-2	0.635
An^+-5-B-11	0.502
$An^†$-(74A)	0

Conidia precultured on YEGCE slants in air were transferred to 5 ml YEGCE (5% glucose) in 150 × 18 cm tubes to 1 × 10^6/ml and incubated for 5 days at 30°C in a Brewer-Allgeier "GasPack" jar set to decolorize methylene blue. Cultures were filtered on a Buchner funnel, and the filtrate was assayed for ethanol with alcohol dehydrogenase (Sigma Chemical Co. method No. 330).

B. *Partial Anaerobiosis*

Initial glucose concn.	Ethanol produced	Theoretical yield	Observed yield
% w/v	*% w/v*	*% w/v*	*% theor.*
20	12	10.2	118
10	4	5.2	78
5	2	2.5	80

Conidia of An^+-2-B-12 precultured on YEGCE slants in air were transferred to 50 ml YEGCE in 125-ml Erlenmeyer flasks to 1 × 10^6/ml and incubated for 21 days at 30°C in an anaerobic oven under nitrogen at atmospheric pressure. Initial oxygen concentration in the medium was 0.2% as determined by a oxygen meter. Final cell dry weight was 0.6–0.9 mg/ml.

Morphogenesis is influenced by oxygen tension. Thus, partial or complete anaerobiosis completely suppresses the formation of aerial hyphae (conidiophores) and conidia on agar slants. Some respiratory-deficient mutants of *Neurospora*, isolated from a facultative anaerobe strain, are incapable of conidiophore and conidial formation in air (21); however, anaerobic culture per se does not select for aconidial or respiratory-deficient mutants. Transfer of anaerobic mycelial slants to air leads to abundant conidial production within a day.

Conidial germination apparently has a more stringent requirement for oxygen than mycelial growth. Thus, facultative anaerobe conidia germinate at low oxygen tension (0.2%) in YEGCE liquid (in contrast to no germination of the obligate aerobe), yet only a low percentage of conidia germinate and grow in complete anaerobiosis in the anaerobic jar method. Thus, in the latter method, growth commences more rapidly and is more reproducible if either large condidial inocula are used or if the conidia are allowed to germinate in air for 8–12 hr before anaerobic culture.

An^+ conidia pregerminated in air, when plated on YEGCE and cultured by the anaerobic jar method for 5–7 days, form microcolonies (1 mm) which macroscopically and microscopically have a yeastlike appearance. 50 microcolonies were transferred to Fries' minimal slants and incubated in air. Within 12–16 hr all colonies reverted to mycelial growth and subsequently formed conidia. Thus, the vegetative growth habit as well as asexual sporulation are physiological adaptations and not due to genetic selection.

Physiological and Enzymological Properties of Facultative Anaerobes

Substantial quantities of ethanol and gas are produced by An^+ mutants cultured on YEGCE in standing flasks in air. Table II, A and B summarizes data on ethanol accumulation in complete and partial anaerobiosis. The total ethanol yield is a function of glucose concentration and approximates the theoretical expectation on the basis of the Embden-Meyerhof pathway of glycolysis (Table II, B).

In some yeasts, such as *Saccharomyces cerevisiae*, glucose represses respiration (the negative Pasteur or Crabtree effect [3]), the formation of electron-

TABLE III

Effect of Culture Medium and Age on Cytochrome Oxidase and Malate Dehydrogenase Activities in a Facultative Anaerobe of Neurospora crassa in Aerobic Culture

Cultrue age	Culture medium*															
	YECE glucose				YECE glycerol				Fries glucose				Fries glycerol			
	COX		MDH		COX		MDH		COX		MDH		COX		MDH	
	Total	SA	Total	SA	Total	SA	Total	SA	Total	SA	Total	SA	Total	SA	Total	SA
hr																
14	55	1.8	400	13	99	1.7	1200	17	3.0	—	300	—	—	—	—	—
24	22	1.6	500	17	12	—	230	—	4.9	2.1	220	20	3.25	—	185	—
30	22	1.3	550	33	12	0.9	370	31	9.3	0.40	270	15	—	—	—	—
37	25	1.3	630	21	6.9	1.0	240	18	13	0.60	250	20	3.15	—	175	—
48	21	1.2	800	25	7.3	1.2	200	18	20	0.80	630	26	3.35	—	170	—

* YECE = yeast extract amino acids basal medium. FRIES = minimal salts medium; glucose and glycerol at 1% w/v.

See Table IV for definition of total enzyme units, specific activity, and culture conditions.

transfer chain enzymes of mitochondrial cristae (22), and the formation of cristal membranes (23). Glycerol, however, being nonfermentable, enforces aerobic respiration and maximal synthesis of respiratory enzymes. Table III summarizes the effect of both carbon source in enriched and minimal media and culture age of An^{+} upon cytochrome oxidase and malate dehydrogenase activities. The results of these experiments indicate a situation unlike that in *S. cerevisiae*. Maximal and least variable activities of cytochrome oxidase are obtained from cultures grown on enriched medium with glucose; yet the cultures are fermentative. There is a slight decline in specific activity of cytochrome oxidase with culture age. Glucose represses total cytochrome oxidase activity at an early culture age on Fries' minimal medium, which is apparently reversed upon utilization of glucose. Growth on glucose, either on enriched or minimal media, yields the same malate dehydrogenase (MDH) activity within the error of assay. Examination of mitochondria *in situ* between 14 and 24 hr reveals that the YECE-glucose cultures have abundant mitochondria with numerous cristae, whereas cells grown on Fries' glucose medium contain fewer mitochondria and these contain few cristae. As stated earlier, Fries' glucose does not permit fermentation or anaerobic growth. Fries' glycerol is a poor medium for germination and growth in aerobic shaker flasks (but is nearly equivalent to Fries' glucose in standing flasks) and yields maximal repression of cytochrome oxidase and malate dehydrogenase. Similarly, on YECE-glycerol, cytochrome oxidase and malate dehydrogenase decline to repressed levels with ageing culture. In addition, either oxygenated media or mechanical agitation apparently prevents glycerol utilization. Moreover, studies of some 120 respiratory-deficient mutants of *Neurospora* (21) indicate that differential growth on glucose and glycerol, either with enriched or basal medium, is not a diagnostic criterion of respiratory deficiency as in *S. cerevisiae* (24).

Repression of Mitochondrial Biogenesis

Repression of mitochondrial biogenesis by anaerobiosis or by inhibition of mitochondrial protein synthesis with chloramphenicol was examined by assays in vitro of mitochondrial enzymes and by observation of mitochondrial morphology *in situ* by electron microscopy.

Others previously established that chloramphenicol specifically inhibits mitochondrial protein synthesis in vivo and in vitro with an obligate aerobic strain of *Neurospora* (25). To determine inhibitory concentrations of the antibiotic, both An^{-} and An^{+} strains were plated on YESSCE plates with various concentrations of chloramphenicol (CAP) and incubated for 2 or 3 days at 30°C. The plates were then overlayed with agar containing a tetrazolium dye, as described in detail elsewhere (21), to determine if the colonies were capable of respiration. With high CAP concentra-

TABLE IV

Effect of Chloramphenicol and Anaerobiosis on Cytochrome Oxidase and Mitochondrial Malate Dehydrogenase Activities in a Facultative Anaerobe of Neurospora crassa

Growth‡ period	Chloramphenicol	Fresh wt§	Enzyme*					
			Cytochrome oxidase			Malate dehydrogenase		
hr	*mg/ml*	*g*	*Total units/g f.w.*‖	*SA*¶	*Relative SA (%)*	*Total units/g f.w.*‖	*SA*¶	*Relative SA (%)*
24	0	1.05 ± 0.05	48.0 ± 6.1	1.38 ± 0.48	100 ± 36	778 ± 48	16.1 ± 2.1	100 ± 13
	2	0.4	13.0 ± 1.3	0.152 ± 0.028	11 ± 2	952 ± 66	12.4 ± 1.6	77 ± 10
	4	0.1	—	—		—	—	
36	0	1.5 ± 0.10	50.8 ± 1.6	1.28 ± 0.08	93 ± 6	878 ± 103	19.3 ± 2.5	120 ± 13
	2	0.9	*4.90 ± 0.54*	*0.099 ± 0.02*	7.7 ± 2	848 ± 60	15.1 ± 2.0	94 ± 12
	4	0.4	*9.20 ± 1.0*	*0.099 ± 0.015*	7.7 ± 2	*1360 ± 95*	13.3 ± 1.7	83 ± 11
48	0	1.75 ± 0.05	53.3 ± 13.4	1.17 ± 0.20	85 ± 17	914 ± 20	15.6 ± 2.0	97 ± 13
	2	1.2	*13.8 ± 1.2*	*0.268 ± 0.054*	23 ± 5	1068 ± 75	18.9 ± 2.5	117 ± 15
	4	0.6	*5.1 ± 0.5*	*0.055 ± 0.070*	4.3 ± 0.9	*1250 ± 71*	13.9 ± 18	86 ± 11
168**	0	0.2	*9.7 ± 0.9*	*0.23 ± 0.05*	18 ± 4	*290 ± 20*	*7.50 ± 0.97*	48 ± 9

* Enzyme units: Cytochrome oxidase, 1 unit = ΔA_{550} mμ of 0.001 per min^{-1}. Malate dehydrogenase, 1 unit = ΔA_{340} min^{-1} of 0.001. Italicized numbers significantly different from no chloramphenicol control at 95% confidence interval.

** Anaerobic culture of An^+ 2B12-3a.

‡ Conidia of a facultative anaerobe mutant (An^+ 2B-12-3a) were inoculated at 5×10^5/ml into 100 ml YECE medium (1% glucose) in a 500 ml florence flask and incubated at 30°C on an Eberbach reciprocal shaker at 150 excursions per min for the period indicated. Chloramphenicol was added to the cultures before inoculation. Anaerobic cultures were incubated in standing flasks of YEGCE (5% glucose) at 30°C in an anaerobic oven under nitrogen. Initial oxygen concentration was 0.2%.

§ Average and standard error of duplicate cultures.

‖ Average and standard deviation of quadruplicate assays of each duplicate culture extract; f.w. indicates fresh weight.

¶ Average and standard deviation. Quadruplicate enzyme assays of each of two culture extracts and duplicate protein determination of each of two extracts.

TABLE V

Ultrastructural Dimensions of Anaerobic and Aerobic Cultured Cells of a Facultative Anaerobe of Neurospora

Culture*	Cell	Average diameter (μ)‡		Av. No. mito. per nucleus	Av. No. cristae per mito.	Av. No. cristae per mitochondrion
		Nucleus	Mitochondrion			
Aerobic	3.5 (33)	1.01 (17)	0.42×0.18 (79)	7.8	447/67	6.7
Anaerobic	2.3 (33)	1.97 (31)	0.82×0.45 (48)	2.0	66/47	1.4

* Facultative anaerobe (An^+ 2B-12-3a) was cultured in YEGCE medium (5% glucose) at 30°C. Aerobic cultures were in 100 ml of medium in a 500 ml Florence flask (at initial inoculum of 5×10^5 conidia/ml) on a rotary shaker for 16–18 hr. Anaerobic cultures were grown in a Brewer-Allgier jar (5 ml culture) for 5–7 days.

‡ Parenthetical values indicate number of figures measured at 30–100,000 final magnification.

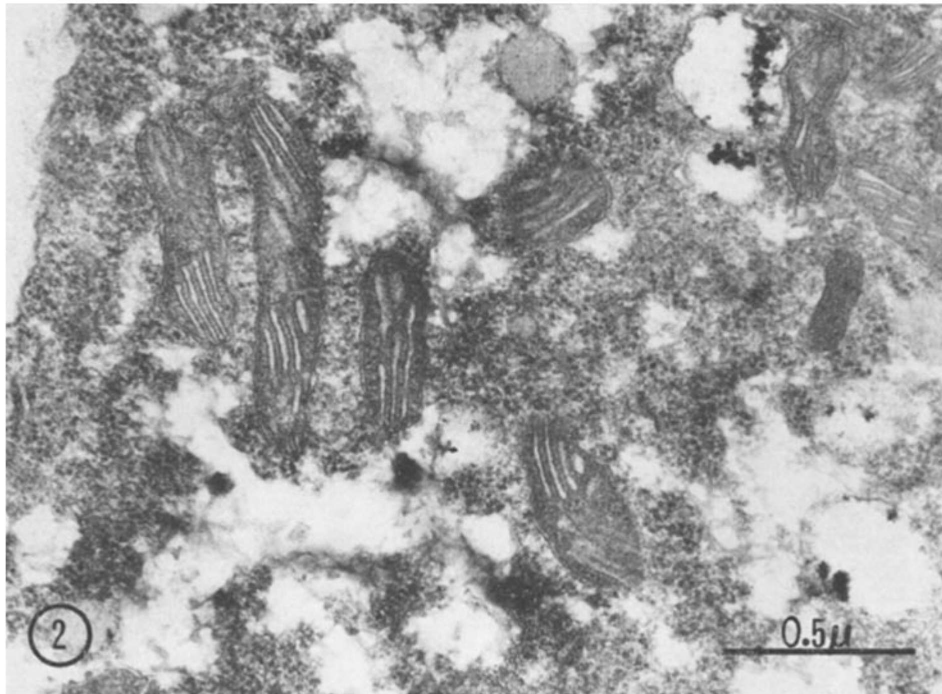
tions (2 or 4 mg/ml), the growth rates of An^+ and An^- strains were inhibited, although the inhibition was less for An^+ strains. Colonies of both strains on CAP plates were respiratory deficient by the tetrazolium test. Such deficiency was reversed when colonies were transferred to media without chloramphenicol; hence, CAP inhibits respiration either directly, or indirectly by inhibiting synthesis of respiratory enzymes, and does not select or induce mutations to respiratory deficiency.

Cultures of An^+ were grown with inhibitory CAP concentrations, and mycelial homogenates were assayed for mitochondrial malate dehydrogenase (MDH) and cytochrome oxidase activities. As summarized in Table IV, the specific activity of cytochrome oxidase was greatly reduced by culture with CAP at two concentrations in all ages of culture. Conversely, CAP led to no significant reduction in MDH activity. Although whole cell homogenates were used, the MDH activity is mitochondrial since, under these conditions, synthesis of the cytoplasmic isozyme of MDH is repressed (15).

Anaerobiosis, as with CAP inhibition, markedly lowers the specific activity of cytochrome oxidase relative to aerobic culture, although not to the same extent as in aerobic culture with CAP. However, in contrast to CAP-inhibited cultures, MDH specific activity was significantly lower in anaerobic culture. Therefore, growth under anaerobic conditions appears to repress both the formation of both cytochrome oxidase, a cristal enzyme, and MDH, a noncristal enzyme; whereas aerobic growth with CAP inhibits only cytochrome oxidase synthesis.

Electron micrographs of thin sections of cells of An^+-2-B-12-3a cultured under aerobic and anaerobic conditions are given in Figs. 2–6. Aerobic cells contain numerous elongate-to-ovate mitochondria with long parallel "orthodox" cristae (Fig. 2). Table V summarizes measurements of some ultrastructural dimensions. In aerobic hyphae, between seven and eight mitochondrial sections per nuclear section and an average of six to seven cristae per mitochondrial section are observed. The cells are further characterized by a granular cytoplasm with occasional single-membrane bounded vesicles having an electron-transparent interior with dense inclusions (Fig. 2). The endoplasmic reticulum is not extensively developed, and usually is represented by short, isolated cisternae with associated ribosomes. Rarely, another type of vesicle is observed which is smaller than mitochondria and has a double membrane surrounding a uniformly dense matrix.

Other experiments indicate that aerobic cultures of An^+ and An^- strains are indistinguishable in ultrastructure. Anaerobic culture of An^+, however, causes marked ultrastructural changes. Generally, anaerobic cells are smaller with enlarged nuclei and mitochondria and contain fewer recognizable mitochondria per nuclear profile. The mitochondria in anaerobic cells are markedly swollen, exhibit fewer and shorter cristae, and contain a dense particulate matrix. A spectrum of cell types in a given anaerobic culture is found, ranging between the extremes shown in Figs. 3–6. A cell such as that in Fig. 4 represents the most extreme type observed, having a grossly enlarged nucleus and a nearly complete absence of mitochondria. Occasionally, small vesicles and, rarely, a rudimentary mitochondrion are pressed between nuclear membrane and plasma membrane. While these studies suggest a reduction in the number of



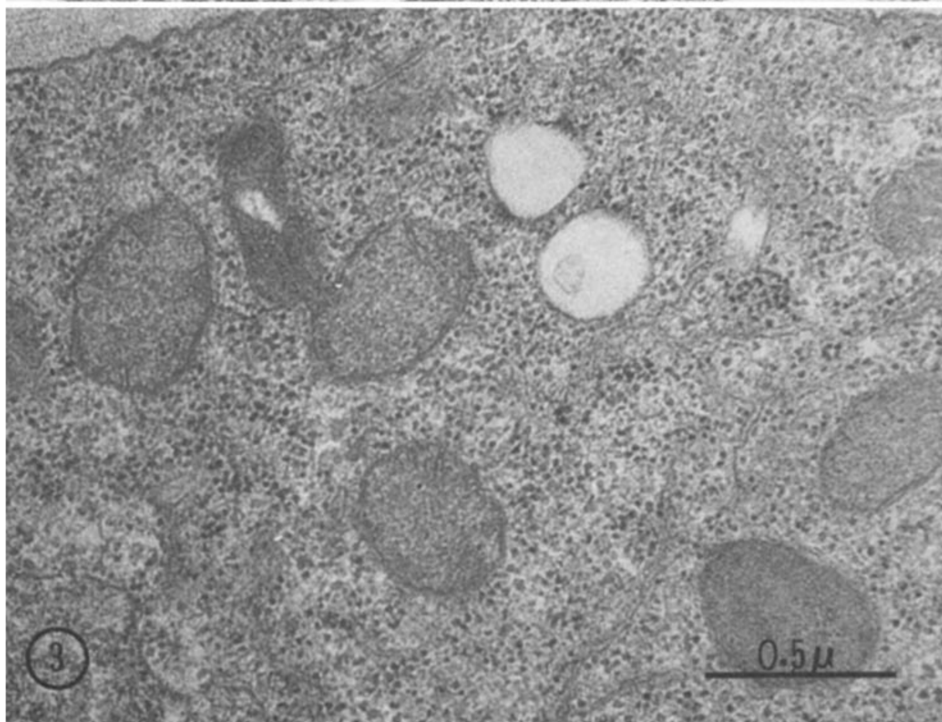


FIGURE 2 Thin section of hypha from aerobic culture in enriched medium. Facultative anaerobe (*An*+-2-B-12-3a) was cultured in shaker flasks at 30°C in YEGCE medium for 18 hr. × 50,000.

FIGURE 3 Thin section of cell from anaerobic culture in enriched medium. Facultative anaerobe (*An*+-2-B-12-3a) was cultured in liquid YEGCE medium at 30°C for 7 days in an anaerobic jar. Aerobic pregermination period was 12 hr. × 50,000.

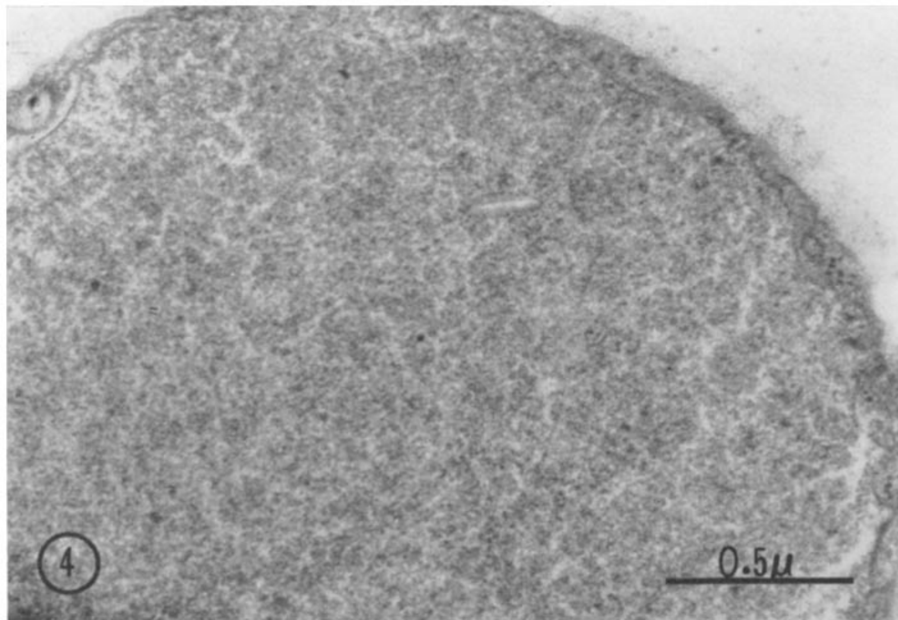


FIGURE 4 Conditions as in Fig. 3.

mitochondria, possible artifacts of fixation or staining cannot be excluded.

Cell structure, such as seen in Fig. 3, is observed infrequently in anaerobic culture. Here mitochondria are enlarged and contain only one or two short cristae in section; however, inner and outer boundary membranes remain. Other membranes, such as plasma membrane, endoplasmic reticulum, unit membrane vesicles (which are probably glyoxysomes [26][1]) and nuclear membrane, resemble those of aerobic cells.

To determine if different times of growth had an effect on the fine structure of anaerobically grown cells, hyphae were grown on solid media in the anaerobic jar for 3 days at 30°C. Since conidia germinate poorly in the anaerobic jar, conidia on the inoculated plates were pregerminated aerobically for either 2 or 6 hr. Thus, while the conditions of anaerobiosis are the same, we were able to obtain cells grown for a shorter time. Differences in the fine structure of these cells compared to those of cells from liquid cultures were found.

When conidia had been germinated for 2 hr aerobically and cultured anaerobically for 3 days, the cellular ultrastructure was severely disrupted. While there was little difference in either the frequency or morphology of mitochondrial profiles, the general appearance of the cells was different. Cells contained numerous electron-transparent regions which were regular in shape but did not appear to have a limiting membrane. Cytoplasmic membrane, resembling smooth endoplasmic reticulum, was abundant and appeared as stacks of cisternae rather than as individual cisternae. The appearance of these cells was not altered by increasing the carbon source in the medium from 1% to 10% glucose.

Hyphal sections derived from conidia germinated aerobically for 6 hr and cultured anaerobically for 3 days were quite similar in appearance to the cells described above, but significant differences were observed. The amount of cytoplasmic membrane was greatly increased, both in amount and in length of membrane cisternae, occasionally extending the entire length of the section (Fig. 6). Whorls of membrane were frequently observed to lie near the cell wall. Mitochondrial profiles were extremely rare and when found were noncristate. Also, the cells occasionally contained paracrystalline inclusions with several types of periodicity (see Fig. 6 for an example). These inclusions were generally quite

[1] Benveniste, K. B. P., C. A. Zuiches, and K. D. Munkres. Unpublished observations.

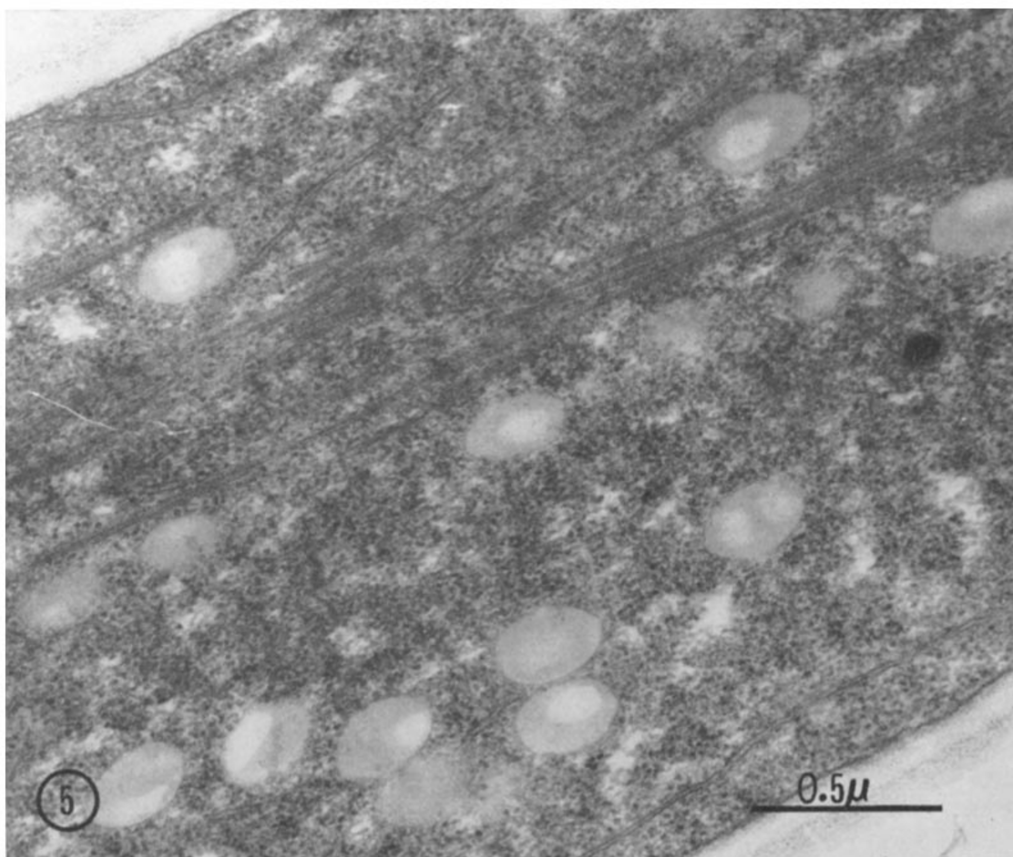


FIGURE 5 Conditions as in Fig. 3 except aerobic pregermination period was 6 hr followed by a 3 day anaerobic incubation. Dialysis membrane method.

large in size. Again, the cellular fine structure was not altered by increasing the carbon source to 10% glucose.

These experiments show that cells grown for a shorter period of time have relatively more free cytoplasmic membrane, appearing both as cisterna-like structures and as whorls. Also, paracrystalline inclusions were observed under these conditions. However, there were no detectable differences in the mitochondrial morphology of cells grown under the various conditions.

Aerobic culture of An^{+} mycelia in the presence of chloramphenicol also leads to degenerate mitochondrial structure (Fig. 7). Such mitochondria, as in anaerobic cells, are enlarged with occasionally only a few short cristae. However, unlike aerobic or anaerobic mitochondria, cristae vary from typical orthodox to enlarged ovate or spherical structures which, in some instances, occupy the majority of the mitochondrial interior, leading to an apparent inversion of the electron-opaque and electron-transparent spaces. In addition, abnormal membranous structures are observed throughout the cytoplasm, although the structures are not the same as those observed in the 3-day anaerobically grown cells. These are probably highly degenerate mitochondria since such structures occasionally contain a cristalike structure on the "inner" membrane. Similar "membrane degeneracy" is also occasionally found in anaerobic cells as well as in respiratory-deficient mutants.[2]

Preliminary experiments with inhibitory concentrations of antimycin A, a respiratory chain inhibitor, suggest that blocking respiration is

[2] Munkres, K. D., N. Howell, and C. A. Zuiches. Unpublished observations.

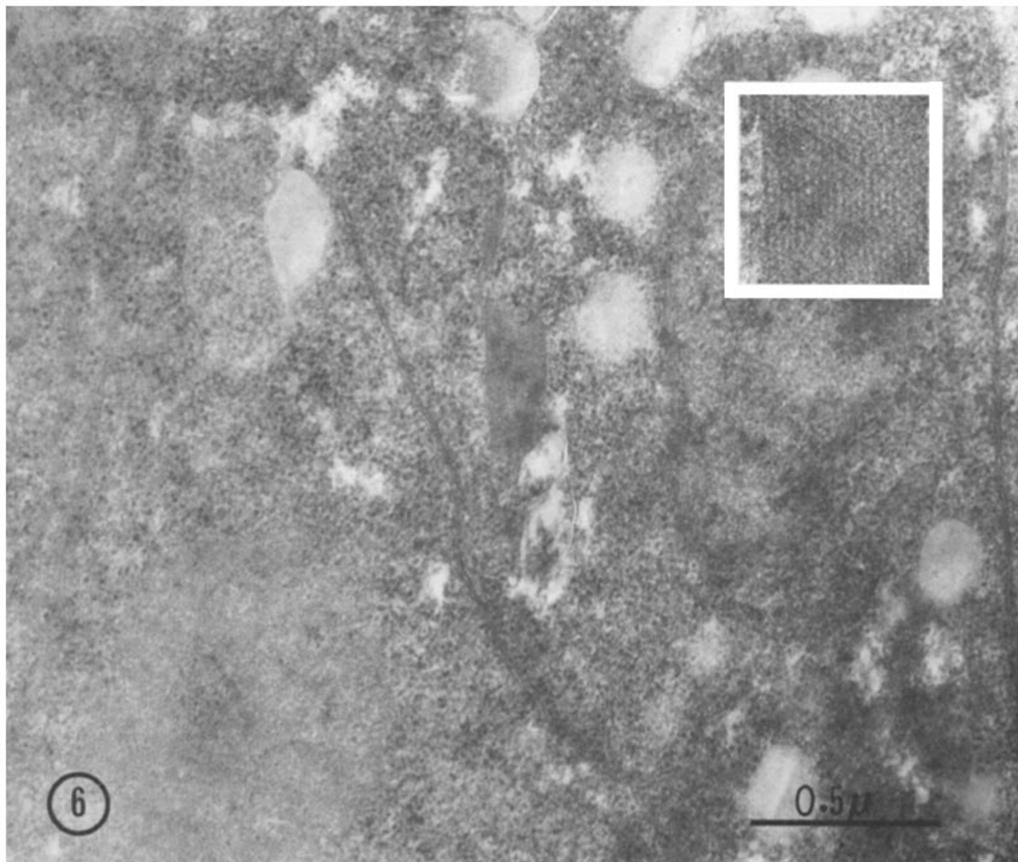


FIGURE 6 Conditions as in Fig. 5. The insert depicts a paracrystalline cytoplasmic inclusion.

sufficient to induce mitochondrial swelling and cristal reduction *in situ*.

DISCUSSION

Possible Nature of the An^+ Mutation

The biochemical nature of the underlying genetic differences between obligate aerobes and facultative anaerobes remains to be discovered; however, the observation of apparent single nuclear gene differences suggests that the basis may be relatively simple. A probable control point in the relative activities of fermentative and oxidative pathways in yeast (27) and in *Neurospora* (7) is the utilization of pyruvate, the branch point of these two pathways.

The degree of fermentation relative to oxidative metabolism is known to influence morphological processes in *Neurospora*. Weiss and Turian (7) concluded that glycolysis and fermentation favor mycelial growth, while oxidative metabolism favors conidiation. The suppression of conidiophore formation and conidiogenesis by partial anaerobiosis observed here points to the same conclusion. In addition, partial anaerobiosis in *Neurospora sitophila* represses perithecial formation to a greater extent than mycelial growth (28).

The Effect of Anaerobiosis on Mitochondrial Configuration

Anaerobiosis, in addition to its effects upon conidiation and colony morphology, also affects cellular ultrastructure, particularly mitochondrial morphology. Weiss and Turian (7) have also noted differences in the *in situ* morphology of mitochondria in mycelia, which utilize a fermenta-

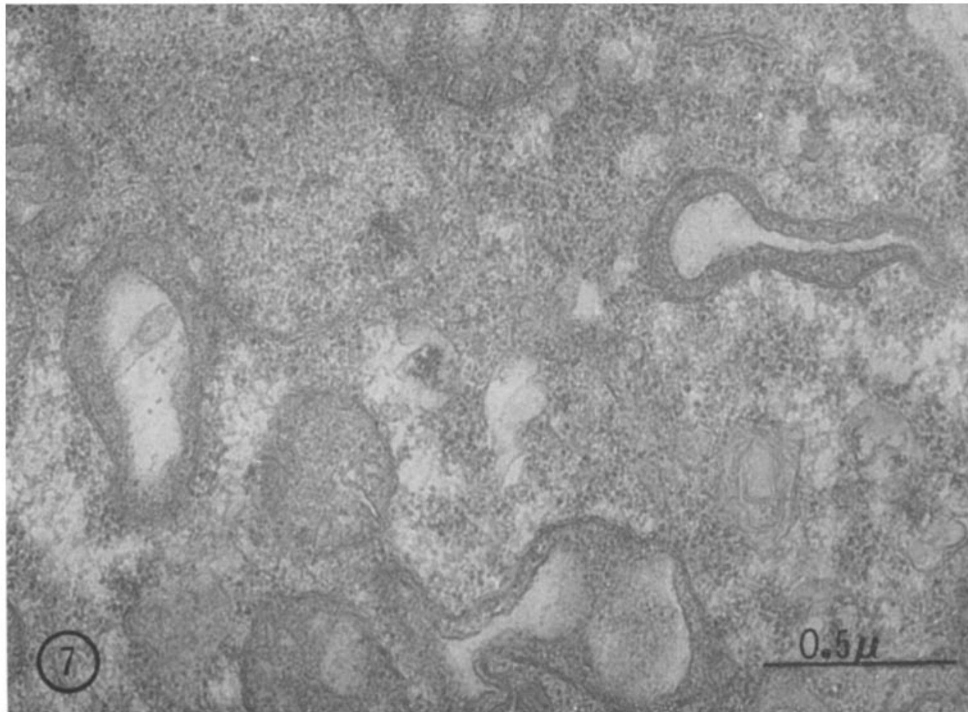


FIGURE 7 Conditions as in Fig. 2 except medium was supplemented with chloramphenicol (4 mg/ml). Note swollen mitochondria with rudimentary infrequent cristae.

tive metabolism to a large extent, and mitochondria in conidia, which rely almost exclusively on an oxidative metabolism. In the present studies, aerobic metabolism and growth of mycelia were inhibited by anaerobiosis, chloramphenicol treatment, and by respiration-deficient mutations (21).[2] Mitochondrial morphology was similar under all conditions, although other cellular structures showed variation among the conditions. Swelling and a paucity of cristal membrane were the principal alterations in the structure of mitochondria of mycelia in which aerobic metabolism was blocked. These alterations were correlated with a reduction in the activity of cytochrome *c* oxidase, an enzyme localized in the cristae (W. J. Cassady, personal communication). In contrast, the specific activity of MDH, an enzyme believed to be localized in the mitochondrial matrix, varies among the conditions used for blocking aerobic metabolism. Hence, while anaerobiosis lowers MDH activity significantly, chloramphenicol treatment does not. These results indicate that cristal membrane synthesis and activity are more dependent upon aerobic metabolism than in the synthesis of a mitochondrial matrix enzyme. Moreover, the effect on MDH specific activity indicates that the effects of anaerobiosis are more complex than the inhibition of mitochondrial protein synthesis. Despite the paucity of cristae and the enlargement of mitochondria in inhibited cells, the inner boundary membrane is persistent. This observation supports the conclusion of others (30–32) that the three mitochondrial membranes (outer, inner, and cristal) show differences not only in function but also in mechanism of biogenesis.

Since conditions sufficient to inhibit respiration (antimycin A treatment) induce swelling and reduce the number of cristae, these observations could indicate that the morphological alterations of mitochondria induced by anaerobiosis, chloramphenicol treatment, or respiratory-deficient mutation may simply be due to the inhibition of respiration. An alternative possibility is that these conditions induce swelling and alteration of cristae by altering the conformation of the membrane subunits as has been suggested by Green and

coworkers (37–39). Presumably, the altered or missing subunits are specific for the cristae, since this mitochondrial membrane shows the greatest dependency on aerobic metabolism. However, the altered cristae may result in changes affecting the whole organelle.

Although a lowered specific activity of an enzyme may reflect a lower rate of synthesis, the activity may also be latent rather than deficient as is found for the cytochrome oxidase activity of an extrachromosomal respiration-deficient mutant of *Neurospora* (40).

Nuclear membranes are similar to mitochondrial membranes in both structure and function such as electron-transport activities, including cytochrome oxidase (41). Moreover, nuclei and mitochondria may have other proteins in common (42). Hence, swelling of nuclei in anaerobically grown cells may operate by a mechanism analogous to that for mitochondrial swelling.

Changes in Cellular Ultrastructure

Anaerobiosis leads to changes in mycelial ultrastructure in addition to those of mitochondria, the most prominent being the accumulation of cytoplasmic membrane under certain conditions of anaerobic growth. Other changes are the presence of electron-transparent regions, paracrystalline inclusions, and fewer ribosomes.

The increase in cytoplasmic membrane also has been observed when *Candida parapsilosis*, an obligate aerobe, is grown under conditions of partial anaerobiosis (31). The accumulation of cytoplasmic membrane has not been reported for *S. cerevisiae*, although all three species show similar alterations of mitochondrial morphology *in situ*.

Moreover, the effect(s) of anaerobiosis on cellular structure is probably more complex than a simple inhibition of mitochondrial protein synthesis since chloramphenicol treatment does not result in the accumulation of membrane in either *Neurospora* or *Candida* (31). Other evidence suggesting that anaerobiosis has a more complex basis was discussed above.

Mycelia arising from conidia pregerminated aerobically for 6 hr, before anaerobic growth, contain more cytoplasmic membrane than those pregerminated for 2 hr. Furthermore, anaerobic growth for 7 days did not induce the formation of either cytoplasmic membrane or paracrystalline inclusions. The basis of these differences is unknown at present. Beck and coworkers (29) have noted that inclusions similar to those observed here can be found in aerobic cultures of *Neurospora* under certain conditions.

While the growth of the fungus *Pythium ultimum*, an obligate aerobe, is sensitive to low doses of chloramphenicol, the drug does not induce gross changes in mitochondrial morphology (45). However, the drug does inhibit the synthesis of cytochromes $a + a_3$ and b and the presence of elementary particles. Possibly, either there are species differences in the contribution of mitochondrial protein synthesis to mitochondrial configuration, or the high levels of drug required for repression in *Neurospora* and *Candida* may be causing secondary effects.

Ultrastructural Variations Among Anaerobically Grown Cells

In anaerobic cultures of *Neurospora*, a heterogeneity of cell types has been found within the same culture and among the various conditions of anaerobic growth. A similar heterogeneity of ultrastructure has been observed in anaerobic cultures of *S. cerevisiae* (10, 46). Some cells are degenerate in appearance with only a swollen nucleus and no observable mitochondria, whereas other cells have nuclei of normal size and rudimentary mitochondria with few or no cristae.

Observations on cultures grown for different periods of anaerobiosis and with differing periods of pregermination suggest that both the culture age and the initial amount of aerobic growth of the culture are important variables in determining cellular ultrastructure.

Schatz and associates (33–35) concluded that during anaerobiosis there is a degeneration of mitochondria to "promitochondria" rather than a loss of mitochondria as suggested by Linnane and coworkers (36). Both groups have also suggested that mitochondrial biogenesis is influenced by the type of media and age of the culture. It now appears that, in yeast, mitochondria in anaerobically grown cells are present but are lipid-depleted and must be fixed with aldehyde rather than permanganate for visualization in the electron microscope (44, 46). Mitochondrial profiles in such cells have also been observed with the freeze-etching technique (35). Here, anaerobic growth of *Neurospora* leads to an apparent decrease in the number of recognizable mitochondria even though the procedure involves aldehyde fixation.

These studies of anaerobic repression in *Neurospora* have not involved the numerous experimental variables utilized for *S. cerevisiae*. However, the absence of glucose repression of mitochondrial biogenesis in *Neurospora* allows a direct examination of the role of oxygen, unlike the situation in *S. cerevisiae* in which both cellular glucose levels and oxygen tension are regulators (43).

Additional investigations of mitochondrial biogenesis and mutation in facultative anaerobes of *Neurospora* are in progress and will be reported elsewhere (21).

The authors thank Doctors D. E. Green, E. F. Korman, and H. Ris for advice and encouragement, and acknowledge the technical assistance of W. Lasher, J. Koplow, K. Connors, and A. Rice.

Paper No. 1373 from the Department of Genetics, published with the approval of the Director of The Agricultural Experiment Station.

This research was supported by grants from The National Institutes of Health (GM-15751), and by an NIH predoctoral fellowship (GM-44231) to Mr. Howell.

A portion of this paper is taken from a dissertation by Mr. Howell submitted to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements of the doctoral degree.

Received for publication 17 November 1970, and in revised form 18 January 1971.

REFERENCES

1. WARBURG, O. 1964. Prefactory Chapter. *Annu. Rev. Biochem.* **33**:1.
2. RAMANO, A. H. 1966. Dimorphism. *In* The Fungi. G. C. Ainsworth and A. S. Sussman, editors. Academic Press Inc., New York. **2**:181.
3. DEDEKEN, R. H. 1966. The Crabtree effect: a regulatory system in yeast. *J. Gen. Microbiol.* **44**:149.
4. WENT, F. A. F. C. 1901. *Monilia sitophila* (Mont.) Sacc., ein technischer Pilz Java. *Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. 2.* **7**: 544, 591.
5. BEADLE, G. W. 1945. Biochemical genetics. *Chem. Rev.* **37**:237.
6. PERLMAN, D. 1950. Observations on the production of ethanol by fungi and yeasts. *Amer. J. Bot.* **37**:237.
7. WEISS, B., and G. T. TURIAN. 1966. A study of conidiation in *Neurospora crassa*. *J. Gen. Microbiol.* **44**:407.
8. KOBR, M. J., D. E. BIANCHI, N. OULEVEY, and G. TURIAN. 1967. The effect of oxygen tension on growth, conidiation, and alcohol production of *Neurospora crassa*. *Can. J. Microbiol.* **13**:805.
9. BEADLE, G. W., and E. L. TATUM. 1945. *Neurospora*. II. Methods of producing and detecting mutations concerned with nutritional requirements. *Amer. J. Bot.* **32**:678.
10. WALLACE, P. G., M. HUANG, and A. W. LINNANE. 1968. The biogenesis of mitochondria. II. The influence of medium composition on the cytology of anaerobically grown *Saccharomyces cerevisiae*. *J. Cell. Biol.* **37**:207.
11. ANDREASEN, A. A., and T. J. B. STIER. 1953. Anaerobic nutrition in *Saccharomyces cerevisiae*. I. Ergosterol requirement for growth in a defined medium. *J. Cell Comp. Physiol.* **41**:23.
12. TATUM, E. L., R. W. BARRATT, and V. M. CUTTER. 1949. Chemical induction of colonial paramorphs in *Neurospora* and *Syncephalastrum*. *Science (Washington)*. **109**:509.
13. BREWER, J. H., and D. L. ALLGEIER. 1966. Safe self-contained carbon dioxide-hydrogen anaerobic system. *Appl. Microbiol.* **14**:985.
14. PERKINS, D. D. 1959. New markers and multiple point linkage data in *Neurospora*. *Genetics.* **44**:1185.
15. BENVENISTE, K., and K. D. MUNKRES. 1970. Cytoplasmic and mitochondrial malate dehydrogenases of *Neurospora*. Regulatory and enzymic properties. *Biochim. Biophys. Acta.* **220**:162.
16. SHERMAN, F., and P. P. SLONIMSKI. 1964. Respiration-deficient mutants of yeast. II. Biochemistry. *Biochim. Biophys. Acta.* **90**:1.
17. BACHMANN, B. J., and D. M. BONNER. 1959. Protoplasts from *Neurospora crassa*. *J. Bacteriol.* **78**:550.
18. FRASCA, J. M., and V. R. PARKS. 1965. A routine technique for double-staining ultrathin sections using uranyl and lead salts. *J. Cell Biol.* **25**:157.
19. SHERMAN, F., and P. P. SLONIMSKI. 1963. Respiration-deficient mutants of yeast. I. Genetics. *Genetics.* **48**:375.
20. WILKIE, D., G. SAUNDERS, and A. W. LINNANE. 1967. Inhibition of respiratory enzyme synthesis in yeast by chloramphenicol: relationship between chloramphenicol tolerance and resistance to other antibacterial antibiotics. *Genet. Res.* **10**:105.
21. HOWELL, N., and K. D. MUNKRES. 1970. Mitochondrial biogenesis in *Neurospora crassa*. III. Induction, isolation and preliminary characterization of respiration-deficient mutants. *Mutation Res.* In press.
22. POLAKIS, E. S., W. BARTLEY, and G. A. MEEK. 1964. Changes in the structure and enzyme activity of *Saccharomyces cerevisiae* in response to changes in the environment. *Biochem. J.* **90**:369.
23. YOTSUYANAGI, Y. 1962. Studies of yeast mitochondria. *J. Ultrastruct. Res.* **7**:121.

24. EPHRUSSI, B. 1953. Nucleocytoplasmic Relations in Microorganisms. Clarendon Press, Oxford.
25. NEUPERT, W., W. SEBALD, A. J. SCHWAB, P. MASSINGER, and T. BUCHER. 1969. Incorporation *in vivo* of ^{14}C-labeled amino acids into the proteins of mitochondria ribosomes from *Neurospora crassa* sensitive to cycloheximide and insensitive to chloramphenicol. *Eur. J. Biochem.* **10**:589.
26. KOBR, M. J., F. VANDERHAEGHE, and G. COMBÉPINE. 1969. Particulate enzymes of the glyoxylate cycle in *Neurospora crassa. Biochem. Biophys. Res. Commun.* **37**:640.
27. HOLZER, H. 1961. Regulation of carbohydrate metabolism by enzyme competition. Cellular regulatory mechanisms. *Cold Spring Harbor Symp. Quant. Biol.* **26**:277.
28. DENNY, F. E. 1933. Oxygen requirements of *Neurospora sitophila* for formation of perithecia and growth of mycelium. *Contrib. Boyce Thompson Inst. Plant Res.* **5**:95.
29. BECK, D. P., G. L. DECKER, and J. W. GREENAWALT. 1970. Ultrastructure of striated inclusions in *Neurospora. J. Ultrastruct. Res.* **33**:245.
30. PENNISTON, J. T., R. A. HARRIS, J. ASAI, and D. E. GREEN. 1968. The conformational basis of energy transformations in membrane systems. I. Conformational changes in mitochondria. *Proc. Nat. Acad. Sci. U.S.A.* **59**:624.
31. KELLERMAN, G. M., D. R. BIGGS, and A. W. LINNANE. 1969. Biogenesis of mitochondria. XI. A comparison of the effects of growth-limiting oxygen tension, intercalating agents, and antibiotics on the obligate aerobe *Candida parapsilosis. J. Cell Biol.* **42**:378.
32. KORMAN, E. F., A. D. F. ADDINK, T. WAKABAYASHI, and D. E. GREEN. 1970. A unified model of mitochondrial morphology. *J. Bioenerg.* **1**:9.
33. CRIDDLE, R. S., and G. SCHATZ. 1969. Promitochondria of anerobically grown yeast. I. Isolation and biochemical properties. *Biochemistry.* **8**:322.
34. POLTAUF, H., and G. SCHATZ. 1969. Promitochondria of anerobically grown yeast. II. Lipid composition. *Biochemistry.* **8**:335.
35. PLATTNER, H., and G. SCHATZ. 1969. Promitochondria of anaerobically grown yeast. III. Morphology. *Biochemistry.* **8**:339.
36. WALLACE, P. G., and A. W. LINNANE. 1964. Oxygen induced synthesis of yeast mitochondria. *Nature (London).* **201**:1191.
37. BLONDIN, G. A., W. J. VAIL, and D. E. GREEN. 1969. The mechanism of mitochondria swelling. II. Pseudo-energized swelling in the presence of alkali metal salts. *Arch. Biochem. Biophys.* **129**:158.
38. BLONDIN, G. A., and D. E. GREEN. 1969. Mechanism of mitochondrial swelling. III. Two forms of energized swelling. *Arch. Biochem. Biophys.* **129**:509.
39. ASAI, J., G. A. BLONDIN, W. J. VAIL, and D. E. GREEN. 1969. The mechanism of mitochondrial swelling. IV. Configurational changes during swelling of beef heart mitochondria. *Arch. Biochem. Biophys.* **129**:524.
40. EDWARDS, D. L., and D. O. WOODWARD. 1969. An altered cytochrome oxidase in a cytoplasmic mutant of *Neurospora. Fed. Eur. Biochem. Soc. Letters.* **4**:193.
41. BEREZNEY, R., L. K. FUNK, and F. L. CRANE. 1970. Nuclear electron transport. I. Electron transport enzyme in bovine liver nuclei and nuclear membrane. *Biochem. Biophys. Res. Commun.* **38**:93.
42. WOODWARD, D. O., and K. D. MUNKRES. 1967. Genetic control, function, and assembly of a structural protein in *Neurospora*. *In* Organizational Biosynthesis. H. J. Vogel, J. O. Lampen, and V. Bryson, editors. Academic Press, Inc., New York. 489.
43. SMITH, D. G., R. MARCHANT, N. G. MAROUDAS, and D. WILKIE. 1969. A comparative study of the mitochondrial structure of petite strains of *Saccharomyces cerevisiae. J. Gen. Microbiol.* **56**: 47.
44. DAMSKY, C. H., W. M. NELSON, and A. CLAUDE. 1969. Mitochondria in anaerobically-grown lipid-limited brewer's yeast. *J. Cell Biol.* **43**: 174.
45. MARCHANT, R., and D. G. SMITH. 1968. The effect of chloramphenicol on growth and mitochondrial structure of *Pythium ultimum. J. Gen. Microbiol.* **50**:391.
46. WATSON, K., J. M. HASLAM, and A. W. LINNANE. 1970. Biogenesis of mitochondria. XIII. The isolation of mitochondrial structures from anaerobically grown *Saccharomyces cerevisiae. J. Cell Biol.* **46**:88.