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Effects of Oxygen and Glucose on Energy Metabolism and Dimorphism of *Mucor genevensis* Grown in Continuous Culture: Reversibility of Yeast-Mycelium Conversion

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Mucor genevensis was grown in both glucose-limited and glucose-excess continuous cultures over a range of dissolved oxygen concentrations (<0.1 to 25 μM) to determine the effects of glucose and the influence of metabolic mode (fermentative versus oxidative) on dimorphic transformations in this organism. The extent of differentiation between yeast and mycelial phases has been correlated with physiological and biochemical parameters of the cultures. Under glucose limitation, oxidative metabolism increased as the dissolved oxygen concentration increased, and this paralleled the increase in the proportion of the mycelial phase in the cultures. Filamentous growth and oxidative metabolism were both inhibited by glucose even though mitochondrial development was only slightly repressed. However, the presence of chloramphenicol in glucose-limited aerobic cultures inhibited mitochondrial respiratory development but did not induce yeast-like growth, indicating that oxidative metabolism is not essential for mycelial development. Once mycelial cultures had been established under aerobic, glucose-limited conditions, subsequent reversal to anaerobic conditions or treatment with chloramphenicol caused only a limited reversal (<35%) to the yeast-like form. Glucose, however, induced a complete reversion to yeast-like form. It is concluded that glucose is the most important single culture factor determining the morphological status of *M. genevensis;* mitochondrial development and the functional oxidative capacities of the cell appear to be less important factors in the differentiation process.

Fungi which exist in two morphological forms provide a useful system in which to study the biochemical basis of vegetative differentiation; the nature of the dimorphic change and its regulation have been the subject of much experimentation and speculation (for reviews, 1-3, 19, 23). Among species of fungi which are dimorphic, the genus *Mucor* has attracted considerable attention. It is known that for some species of this genus culture conditions which favor fermentation also promote yeast-like morphology, whereas oxidative conditions promote mycelial development (5, 9, 14, 23, 24).

To gain a better insight into the molecular processes underlying the transformation from yeast-like to mycelial forms, a more precise understanding of the transition from fermentative to oxidative metabolism is required. Furthermore, a knowledge of the extent to which transformation between the two differentiated states is reversible would be most useful in determining the nature of the underlying biochemical events, in the same way that "commitment" has been useful in an analysis of bacterial sporulation and slime mold aggregation and differentiation (6, 22).

An examination of the physiological regulation of differentiation in dimorphic fungi and the reversibility of the mechanism involved would be most meaningful if carried out under conditions in which environmental parameters are controlled and maintained constant over long periods of time. Continuous culture in a chemostat provides these conditions, since a single parameter at a time can be varied, and each culture regime can be sustained so that the organism comes to effective equilibrium with its environment. By utilizing constant dilution rates, cells can be compared at constant growth rate, and the transition from and return to a basal condition can be achieved quite precisely.

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In batch cultures, by contrast, it is frequently difficult to distinguish between effects due to intrinsic changes in the gaseous environment, in the concentration of nutrients, and in the growth rate.

In this communication we describe experiments with *Mucor genevensis* grown in continuous culture which relate quantitatively the extent of differentiation between yeast and mycelial phase with physiological and biochemical parameters reflecting the fermentative and oxidative capacities of the cell. In addition, it is shown that, once mycelial cultures are established under high aeration conditions, subsequent reversal to yeast-like morphology can be achieved only by providing excess glucose in the medium; anaerobiosis results in only a partial reversal if excess glucose is not present.

MATERIALS AND METHODS

Organism. *M. genevensis* NRRL 1407 was maintained on cornmeal agar slopes (Difco Emerson's media) at 12 or 4 C and transferred every 8 weeks. Spores were harvested from these slopes as described previously (14).

Liquid culture medium. The nutrient used to maintain the chemostat cultures contained (per liter): 10 to 60 g of glucose, 3.0 g of $(NH_4)_2SO_4$, 1.0 g of KH_2PO_4, 0.5 g of NaCl, 0.1 g of $CaCl_2 \cdot 2H_2O$, 2.0 g of $MgSO_4 \cdot 7H_2O$, 5.4 mg of ferric citrate $\cdot 3H_2O$, 5.0 g of yeast extract (Difco), and 5 ml of a trace metal solution. The trace metal solution contained per 5 ml: 0.2 mg of $CuSO_4 \cdot 5H_2O$, 0.5 mg of KI, 0.5 mg of $ZnSO_4 \cdot 7H_2O$, 0.5 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.5 mg of $Na_2B_4O_7 \cdot 10H_2O$, and 1.0 mg of $MnSO_4 \cdot 4H_2O$.

Continuous culture. Cells were cultured in either a New Brunswick (U.S.A.) Microferm fermenter equipped with pH and dissolved oxygen controls or in a Gallenkamp controlled environment culture apparatus (U.K.). Mackereth oxygen electrodes supplied by EIL (U.K.) or the autoclavable version prepared as described by Borkowski and Johnson (8) were used in these experiments. The EIL electrode was sterilized with a gas mixture containing ethylene oxide (Sterigas, Commonwealth Industrial Gases, Sydney, Australia). The electrodes were calibrated against solutions equilibrated either with standard gas mixtures containing 1 or 5% oxygen in nitrogen (Commonwealth Industrial Gases, Sydney), or with lower oxygen tensions prepared by dilution of these gas mixtures with nitrogen using calibrated flowmeters (Roger Gilmont Instruments, U.S.A.). Dissolved carbon dioxide concentration was measured with a Radiometer (Copenhagen) carbon dioxide electrode connected to a Radiometer pH meter PM26. This electrode was also sterilized with ethylene oxide. The chemostat was operated at a dilution rate of 0.06 h^{-1} at 28 C. Approximately 10^6 spores were inoculated into the growth chamber, the culture was grown batch-wise overnight, and the continuous mode of operation was then commenced.

Samples of cells were collected directly from the culture vessel, and weights were determined after drying the samples at 100 C for 24 h.

Potential respiration, which is the respiratory rate at saturating concentrations of oxygen, was measured by using cells resuspended in 0.05 M potassium phosphate buffer (pH 7.0) containing 0.02 M glucose; the oxygen exhaustion curve was recorded by using a Yellow Springs Instruments oxygraph and a Clark-type oxygen electrode. The sensitivity of this respiration to cyanide was measured by adding potassium cyanide (2 mM) after the glucose. Actual respiration (Q_{O_2}, milliliters of oxygen per gram dry weight per hour) is the rate of oxygen consumption under steady state conditions in the chemostat, and was determined by monitoring the oxygen concentration in both the inlet and outlet gas streams and from the difference calculating the respiration rate of the total culture (21).

Carbon dioxide release. Q_{CO_2} (milliliters of carbon dioxide per gram dry weight per hour), the specific evolution of carbon dioxide in situ by chemostat culture, was measured by using a similar gas balance technique (21).

Ethanol and glucose determinations. Ethanol in cell-free culture filtrates was determined by using the assay kit supplied by Sigma Chemical Co., and glucose was estimated with the glucostat reagent (Worthington Biochemicals). The ethanol production by steady state cultures has been expressed in terms of b_e (moles of ethanol produced per mole of glucose consumed).

Preparation of cell-free homogenates. After being washed with water and 0.9 M sorbitol, 10 mM tris(hydroxymethyl) aminomethane (Tris) (pH 7.0) and 5 mM dipotassium ethylenediaminetetraacetate (EDTA) at 2 C, packed cells were resuspended in an equal volume of this buffer. An equal volume of glass beads was added to the cell suspension and the slurry was shaken at top speed for 25 s in a Braun MSK homogenizer, with carbon dioxide as a coolant. Cell debris and glass beads were removed by successive centrifugation at 2,000 $\times$ *g* and 2,500 $\times$ *g* for 5 min at 2 C. The activities of enzymes in these homogenates were assayed as soon as practicable.

Enzyme assays. The procedures for the assay of respiratory enzymes have been described previously (20, 21). Isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2) were assayed by the methods of Dixon and Kornberg (11). Glycolate oxidase (EC 1.1.3.1) was assayed by the method of Feierabend and Beevers (13).

Protein was determined by the method of Lowry et al. (17).

Cytochrome spectra. Cytochromes were determined at 77K by using a Cary 14R spectrophotometer fitted with a scattered-light transmission accessory. Cell-free homogenates were examined and the sample cuvette was reduced with either sodium succinate in the presence of potassium cyanide or with dithionite. The reference cell was oxidized with a few grains of ammonium persulphate.

Extraction of fatty acids and sterols. The procedures used were those described by Gordon, Stewart, and Clark-Walker (14).

Determination of yeast-mycelium ratio. The pro-

portion of yeast-like cells in cultures was determined by differential filtration. To retain the mycelia, culture samples (not exceeding 30 mg dry weight) were first filtered through preweighed disks of nylon net (L.K.B. support net, code no. 0655). The mycelia caught on the net were carefully resuspended in about 10 ml of water and refiltered through the net to ensure removal of the yeast cells. This washing was repeated three times. The yeast cells remaining in the filtrate were collected and washed on preweighed glass fiber filters (Whatman). Both the net and the glass fiber filters were then dried at 105 C for 24 h and then weighed. The effectiveness of the method in separating the two morphological types is illustrated in Fig. 1a and b.

RESULTS

Physiology of glucose-limited cultures. The effects of oxygen on the energy metabolism and dimorphic transition of *M. genevensis* were studied in glucose-limited continuous cultures. As the dissolved oxygen concentration was increased from 0.1 to 12.5 μM, the morphology changed from yeast-like to highly branched mycelial clumps (Fig. 1c to f). The relative proportions by weight of mycelial and yeast-phase cells at different oxygen concentrations are shown in Fig. 2c.

Concomitant with the change in morphology, there was a shift from fermentative to oxidative metabolism (Fig. 2a to d and Table 1). Under micro-aerobic conditions the cell yield was low but increased about fivefold as the oxygen concentration was raised to 15 μM. A b_e value of 1.9 (moles of ethanol produced per mole of glucose) was found for the most anaerobic cultures, and this ratio declined with increasing oxygen concentration so that in aerobic cultures ethanol was barely detectable. The actual respiration rate also increased with increasing oxygen tension, whereas the respiratory quotient RQ (Q_{CO_2}/Q_{O_2}) approached 1 as conditions generating maximum cell yield were approached (Fig. 2b and c). Dissolved carbon dioxide concentration at steady state in the cultures was found to decline slightly as the dissolved oxygen concentration was increased. Under microaerobic conditions the carbon dioxide concentration was 19 mM, and this declined to 12.5 mM when the dissolved oxygen concentration was raised to 15 μM.

The increase in the actual oxygen consumption of the culture was somewhat less sensitive to environmental oxygen tension than was the increase in cell yield (Fig. 2a and b). The potential respiration rate, on the other hand, increased to a maximum at even lower oxygen concentrations than did cell yield (Fig. 2d). Furthermore, the apparent K_m for oxygen as calculated from the exhaustion curves used to measure potential respiration increased as the oxygen concentration at which the cells were cultured was increased (Fig. 3). The cyanide-insensitive portion of the potential respiration rate (Fig. 2d) displayed a maximum value in cells grown at about 1.0 μM oxygen; at higher concentrations this pathway accounted for only minor amounts of respiration in these cells.

Respiratory enzyme synthesis in response to oxygen. To determine which of the respiratory rates (actual or potential) most closely reflected the activity of the respiratory enzyme complexes in the cells, representative respiratory enzymes were assayed in cell-free homogenates. Cytochrome *c* oxidase, succinate-cytochrome *c* reductase, and succinate dehydrogenase activities in cell-free homogenates were maximal in cells grown at 2.5 μM oxygen (Fig. 4); the activities of the latter two enzymes declined at higher oxygen concentrations. Catalase and cytochrome *c* peroxidase (enzymes associated with hydrogen peroxide decomposition) required higher concentrations of oxygen for maximum development; neither showed any significant decline at the higher oxygen concentrations. Isocitrate lyase activity was maximal in cells grown at 1.0 μM oxygen decreasing in cells grown at higher oxygen tensions. Malate synthase and glycolate oxidase could not be detected in cells grown under any of these conditions, although the presence of these enzymes can be readily demonstrated in *Saccharomyces cerevisiae* grown under similar conditions using the same assay procedures (21).

Cytochrome formation in cells grown over a range of oxygen concentrations up to 25 μM is shown by the difference spectra of cell-free homogenates recorded at liquid nitrogen temperature (Fig. 5). In aerobic cultures absorption maxima occurred at 605, 560, and 548 nm, corresponding to the α-bands of an *a*-type cytochrome, a *b*-type cytochrome, and a *c*-type cytochrome, respectively. Cells cultured microaerobically (0.1 μM oxygen) lacked a recognizable 560-nm band but still contained an absorption band at 548 nm, and significant amounts of the *a*-type band. Additional bands were evident at 553 and 558 nm, and a shoulder occurred at approximately 546 nm. As the dissolved oxygen concentration increased, the 560-nm band appeared and could be resolved from the band at 558 nm. The inability to resolve a 553-nm band in aerobic cultures may be due to the masking effect of the strong absorption at 548 nm; asymmetry of the spectrum in the region of 553 nm is consistent with this possibility.

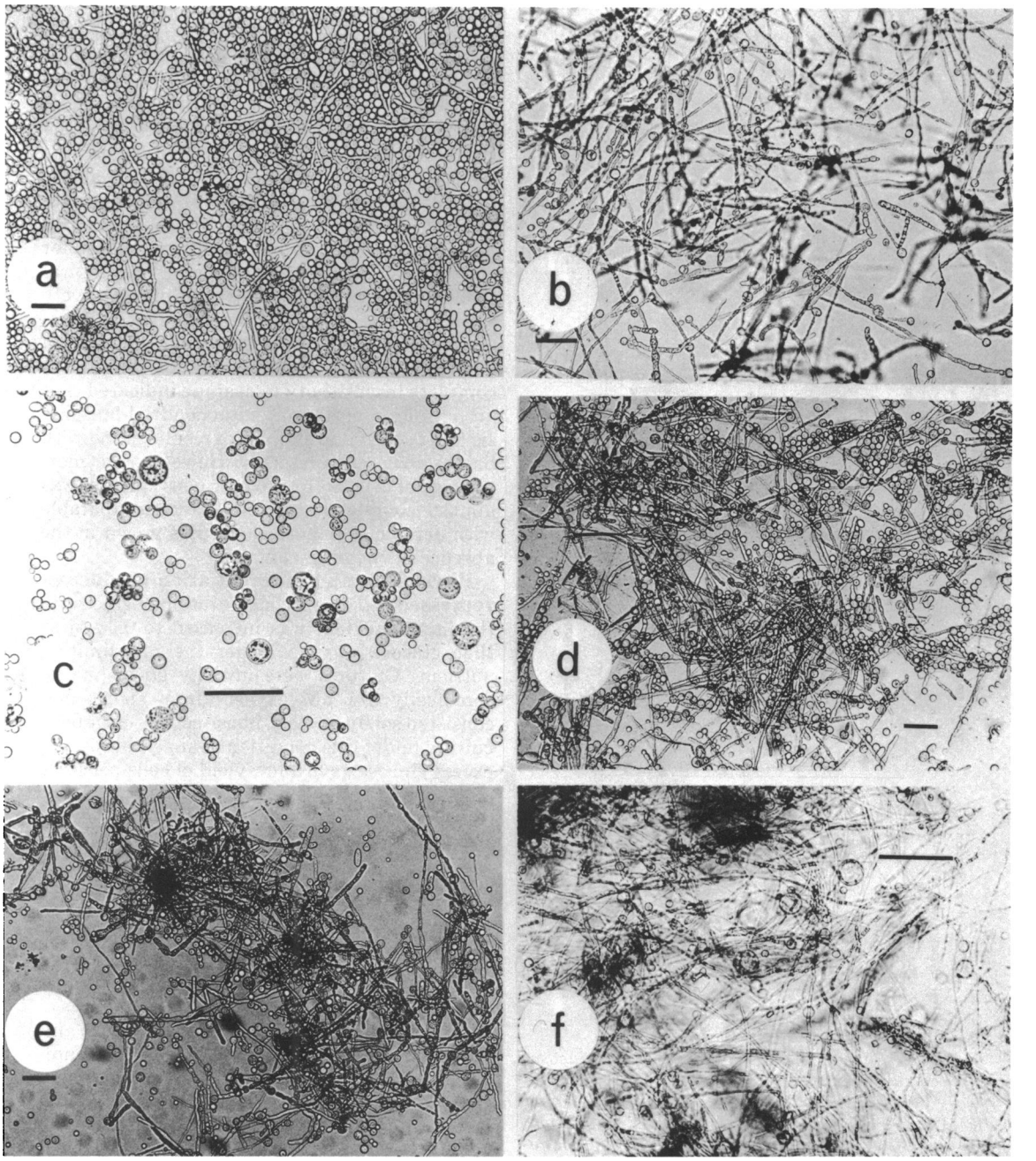

FIG. 1. *Yeast-like and mycelial phases separated by differential filtration. Photomicrographs (a) and (b) illustrate the effectiveness of the enrichment of the two morphological types by using this technique. The original culture was grown at 2.5 μM dissolved oxygen concentration, (c) to (f). The effect of dissolved oxygen concentration on the morphology of glucose-limited continuous cultures grown: (c) at 0.1 μM oxygen, (d) at 2 μM oxygen, (e) at 4.5 μM oxygen (f) at 12.5 μM oxygen. The bar represents 50 μm.*

Fatty acid composition of cells from glucose-limited cultures. In chemostat cultures of *S. cerevisiae*, another facultatively anaerobic eukaryote, the development of mitochondrial respiratory complexes, and other oxidative functions appear to be closely related to an in-

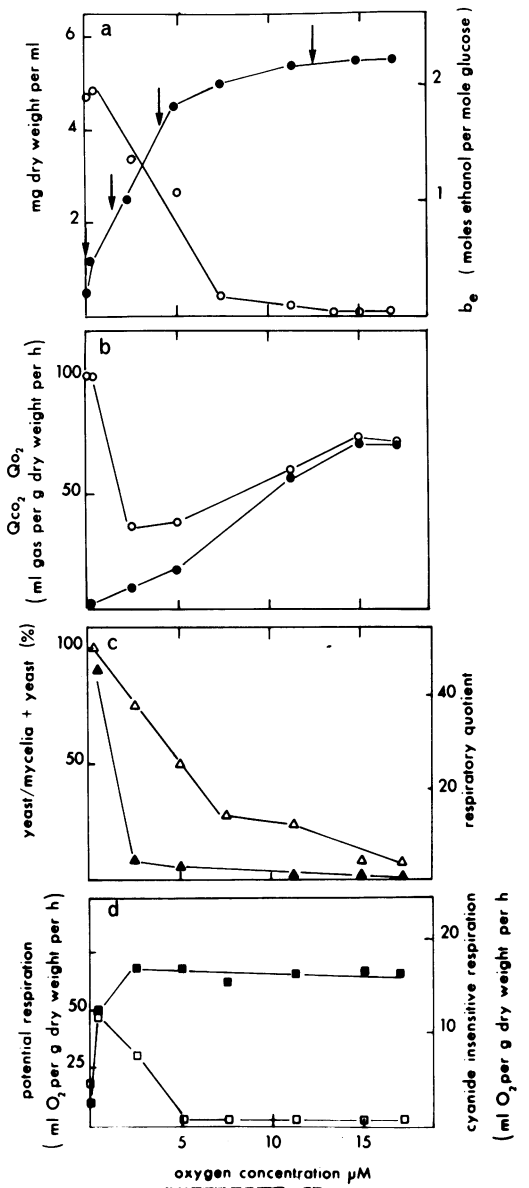

FIG. 2. *Physiological parameters of glucose-limited cultures: (a), (●) dry weight; (○) b_e; (b), (○) Q_{CO_2}, (●) Q_{O_2}; (c), (△) yeast/yeast + mycelia (wt/wt %), (▲) RQ (Q_{CO_2}/Q_{O_2}); (d), (■) potential respiration, (□) potential respiration insensitive to cyanide. Photomicrographs of samples corresponding to the arrows in (a) are presented in Fig. 1c to f. The input nutrient contained 26 mM glucose and the steady state concentration was <25 μM.*

crease in the amount and proportion of unsaturated fatty acids in the cells (20, 21).

The fatty acid composition of *M. genevensis* grown in glucose-limited conditions is shown in Table 2. The results show that as the oxygen concentration is increased the total fatty acid concentration increases and the proportion of saturated fatty acids, both short and long chain, decreases. The proportion of unsaturated fatty acids, particularly 18:2 and 18:3, increases greatly; in fact, almost all of the increase in the amount of fatty acids seen in the more aerobic cells is accounted for as unsaturated fatty acids.

Effect of chloramphenicol on glucose-limited cultures. When chloramphenicol (9 mM) was present in aerobic cultures limited for glucose, culture parameters such as b_e, respiration, and carbon dioxide evolution were very similar to those obtained for anaerobic cultures grown in the absence of the antibiotic (Table 1). The low potential respiration and its almost complete insensitivity to cyanide indicated that an inhibition of normal mitochondrial biogenesis had occurred in support of results found with batch-grown cultures (10). However, the morphology of cells in these continuous cultures was almost completely mycelial and comparable with aerobic filamentous cultures grown in the absence of the drug (Fig. 1f).

Physiology and differentiation of glucose-repressed cells. The concentration of glucose in the input nutrient was increased to 0.33 M so that glucose was no longer the rate-limiting nutrient. Cultures were initially grown microaerobically (0.1 μM oxygen) from spores and consisted solely of yeast-phase cells. Continuous culture under this condition of anaerobiosis and excess glucose gave a low yield of cells per mole of glucose (Table 1), comparable to that obtained under glucose-limited conditions of microaerobic growth. Carbon dioxide evolution was high (Fig. 6b) and G_{CO_2} (moles of CO_2 evolved per mole of glucose consumed) was calculated to be 0.9, compared with 1.6 for microaerobic, glucose-limited cultures. The actual respiration rate was very low, although potential respiration was half that found in glucose-limited cultures.

When the oxygen concentration was increased to 25 μM under excess glucose conditions, actual respiration remained low at about one-eighth of the potential respiration rate (Fig. 6b and c). Carbon dioxide evolution in these aerobic, excess glucose cultures was almost double the value obtained under anaerobic conditions (Fig. 6b) and G_{CO_2} had increased to 2.2, suggesting that all of the glucose was being metabolized glycolytically. Ethanol production decreased as conditions became more aerobic, but b_e declined only slightly, from 1.4 to 1.0 (Fig. 6a). In contrast with glucose-limited cultures the increase in potential respiration rate paralleled the increase in cell yield (Fig. 6a and

TABLE 1. *Physiological parameters of continuous cultures of M. genevensis*

Steady state conditions		Yield (mg dry wt per mmol of glucose)	b_e (moles of ethanol per mole of glucose)	Respiration (ml of gas per g dry wt per h)		
Oxygen (μM)	Glucose[a] (mM)			Potential	Q_{O_2}	Q_{CO_2}
Glucose limited						
0.1	<0.025	20	1.9	13 (50)[b]	<2	104
15	<0.025	227	0.01	67 (2)	68	72
Glucose limited + chloramphenicol[c]						
20	<0.025	56	1.5	7 (85)	<2	120
Glucose repressed						
0.1	215	14	1.4	13 (50)	<2	83
12	105	19	1.0	34 (3)	5	156

[a] The concentration of glucose in the input nutrient was 25 mM for glucose-limited cultures, 340 mM for glucose-repressed cultures.
[b] Potential respiration insensitive to cyanide (%).
[c] 9 mM.

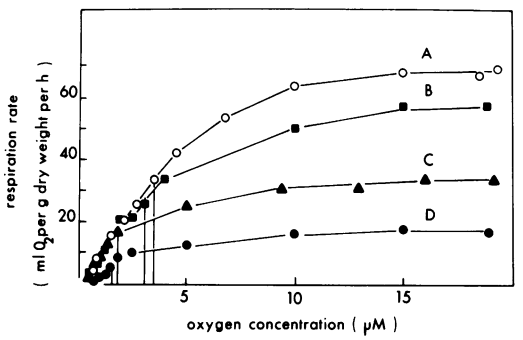

FIG. 3. *Effect of oxygen concentration on the respiratory activity of cells removed from glucose-limited cultures. The cultures were grown at dissolved oxygen concentrations of: (A) 7.5 μM, (B) 2.5 μM, (C) 0.5 μM, (D) <0.1 μM. Lines indicate the apparent K_m values for the potential (i.e., maximal) respiration rates.*

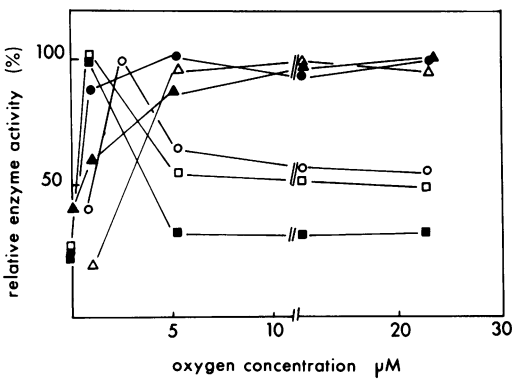

FIG. 4. *Enzyme activities of cell-free homogenates from glucose-limited cultures grown at oxygen concentrations between 0.1 to 25 μM. Symbols: ▲, catalase; △, cytochrome c peroxidase; ●, cytochrome c oxidase; □, succinate cytochrome c reductase; ■, succinate dehydrogenase; ○, isocitrate lyase. The enzyme activities have been expressed as a percentage of the maximal activities observed. The maximal activities expressed as initial reaction rates (micromoles of substrate per minute per milligram of protein) were: catalase, 72; isocitrate lyase, 0.02; succinate dehydrogenase, 0.15; cytochrome c peroxidase, 0.1; succinate cytochrome c reductase, 0.12; cytochrome c oxidase, 0.15.*

c); the proportion of potential respiration insensitive to cyanide showed a maximum in cells grown at 2 μM oxygen and thereafter declined.

The difference spectra of glucose-repressed cells showed a decrease in absorption bands at 603 and 560 nm relative to cells grown at comparable oxygen concentrations but in the glucose-limited condition. The unsaturated fatty acid analysis (Table 2) of these cells was almost identical to that of cells from glucose-limited cultures. Essentially similar results were obtained for aerobic mycelial cultures and yeast-like cultures induced aerobically with phenethyl alcohol (14).

High glucose concentration repressed the development of mycelial forms in the cultures. At oxygen concentrations up to 75 μM, yeast-like morphology persisted in glucose-repressed cultures, although certain differences were evident compared with glucose-limited conditions. Firstly, the cells were larger (Fig. 7a) than those observed in comparable experiments under glucose-limited microaerobic conditions (30 to 80 μm cf. 10 to 20 μm); secondly, after prolonged growth (14 days) at high glucose concentration lysis of some cells occurred (Fig. 7b), and an accumulation of cells bearing stunted mycelial projections was evident (Fig. 7c).

Reversibility of the dimorphic change. The effects of microaerobic conditions (0.1 μM oxy-

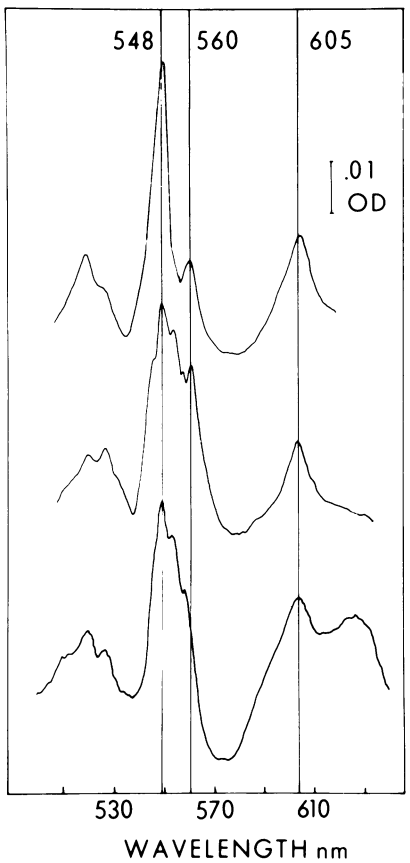

FIG. 5. *Reduced versus oxidized difference spectrum at 77 K of cell-free homogenates from glucose-limited cultures grown at 10 μM oxygen (top trace), 1.0 μM oxygen (middle trace), and <0.1 μM oxygen (lower trace).*

gen), excess glucose, and chloramphenicol on the morphology of steady state, mycelial cultures were examined to test the reversibility of the dimorphic response. Mycelial cultures were generated and equilibrated at high oxygen concentrations under glucose-limited conditions. The imposition of microaerobic conditions on these cultures did not cause complete reversion of the culture to yeast-like morphology even after 28 days. Approximately one-third of the culture consisted of yeast-like cells; the remainder were highly-branched mycelia (Table 3). Carbon dioxide evolution and ethanol concentration were high and the oxygen consumption of the cultures was very low. Thus the reversibility was complete as judged by these metabolic parameters (cf. Table 1 and Table 3); morphologically, however, the reverse transition was incomplete. There was no effect, even after 20 days, on the proportion of the two morphological forms in these microaerobic cultures when carbon dioxide was substituted for nitrogen as the sparging gas.

High glucose concentrations, on the other hand, caused complete reversion of the mycelial cultures to yeast-like morphology. However, as shown in Fig. 8c and d, the cells aggregated together to form characteristic clusters. These are to be compared with the yeast-like cultures cultivated from spores in excess glucose (Fig. 8a and b). The physiological parameters (Table 3) were found to be generally similar to those just described for the anaerobic, derepressed culture (Table 3).

When chloramphenicol was added to the cultures via the nutrient feed to give concentrations of the antibiotic in the culture vessel of 12 mM, there was no significant reversion to single cell morphology when cultures were maintained at 70 μM oxygen (Table 3), even after prolonged growth (28 days). In most respects, the physiological parameters measured in these cultures were similar to the values found in anaerobic, derepressed cultures described in Tables 1 and 3.

DISCUSSION

The experiments described in this communication were devised to examine, under conditions of continuous culture and thus constancy of environment, firstly, to what extent oxidative metabolism can be correlated with cellular morphology in *M. genevensis;* secondly,

TABLE 2. *Fatty acid contents of M. genevensis as a function of dissolved oxygen concentration*

Glucose concn (mM)	Oxygen concn (μM)	Total fatty acid (mg per g dry wt)	Fatty acid distribution (weight %)		
			C_{10}–C_{14}	C_{16}–C_{18}	$C_{18:1,\ 18:2,\ 18:3}$
0.025	Anaerobic	25	25.5	47.3	27.2
0.025	1.25	36	26.8	20.5	54.2
0.025	2.8	70	21.5	7.8	70.7
0.025	5	65	4.7	7.7	87.6
0.025	20	78	4.5	19.4	76.1
120	25	101	4.8	19.7	75.5

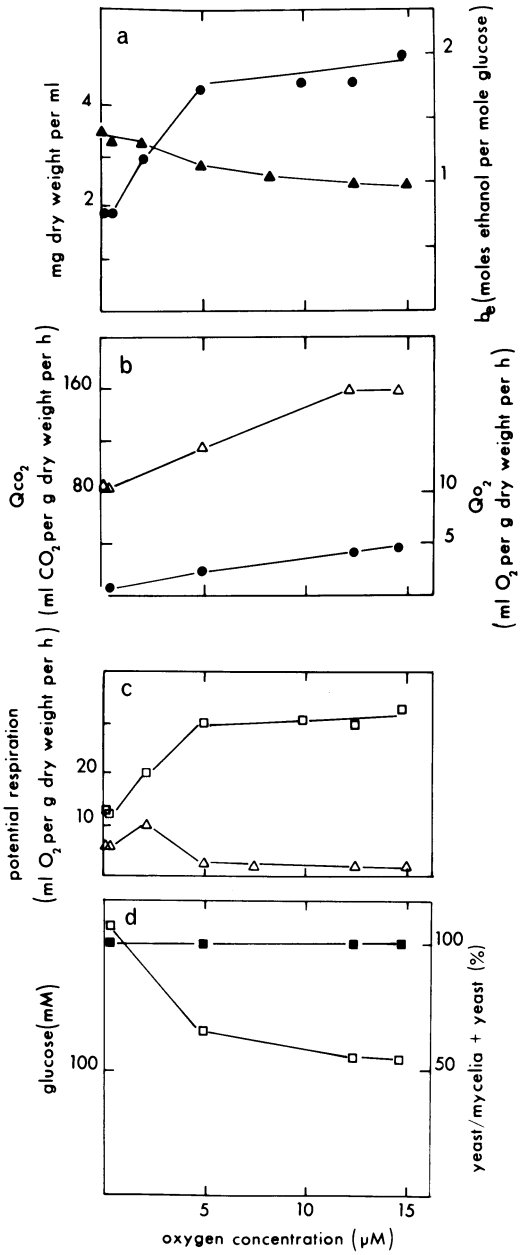

FIG. 6. *Physiological parameters of cultures grown in excess glucose: (a), (●) dry weight; (▲) b_e; (b), (△) Q_{CO_2}, (●) Q_{O_2}; (c), (□) potential respiration, (△) potential respiration insensitive to cyanide; (d), (■) yeast/yeast + mycelia (wt/wt %), (□), steady state glucose concentration. The input nutrient contained 340 mM glucose.*

whether under conditions of continuous culture the yeast/mold dimorphism exhibited by this organism is reversible.

With regard to oxidative metabolism and morphology, two processes are responsive to oxygen. Firstly, oxygen at low concentrations acts to initiate the development of the respiratory apparatus of the cell. Thus, at dissolved oxygen concentrations as low as 2.0 μM the potential respiration of the cells is maximal. This respiratory development occurs concomitantly with an increase in activity of enzymes of the mitochondrial respiratory chain, in particular cytochrome *c* oxidase. However, as judged by parameters such as cell yield, ethanol concentration, actual respiration rate, carbon dioxide release, and respiratory quotient, metabolism at 2 μM dissolved oxygen is essentially fermentative. This may be a consequence of the oxygen concentration under these conditions being insufficient to saturate the oxidase system (for which the apparent K_m for oxygen is approximately 2 μM in cells grown under microaerobic conditions).

Although at higher oxygen concentrations there is no further change in cytochrome *c* oxidase activity, the significant decline in activity of other mitochondrial enzymes is similar to that seen in certain bacteria, in which there is apparently an optimal environmental oxygen concentration for the synthesis of the respiratory apparatus (26). This contrasts with the situation in other eukaryotes such as yeast, where there is no decline in respiratory activity or related enzymes once a saturating oxygen concentration is reached (16, 18, 21).

The second effect of oxygen occurs at higher concentrations (5 to 10 μM). Both the relative proportions of yeast and mycelial forms in the culture and the development of respiratory metabolism (as distinct from respiratory potential) change approximately in parallel in cells proceeding towards more aerobic conditions of growth. In this region of aeration, oxygen concentrations are sufficient to saturate the oxidase system, and the increase in respiratory quotient and cell yield, and the decline in ethanol concentration, are physiological manifestations of this and related changes in these cells.

There is evidence to suggest that yeast extract promotes mycelial development in some strains of *M. rouxii* even under anaerobic conditions (1, 15). This was not our experience with *M. genevensis* in liquid medium. In fact, excess yeast extract was added to the input nutrient so that the growth of microaerobic cultures would be limited by glucose and not by anaerobic growth factors (4, 12). In the absence of yeast extract growth was so slow that steady state microaerobic cultures could not be established. Although in glucose-limited cultures other essential nutrients are in excess, subtle changes in

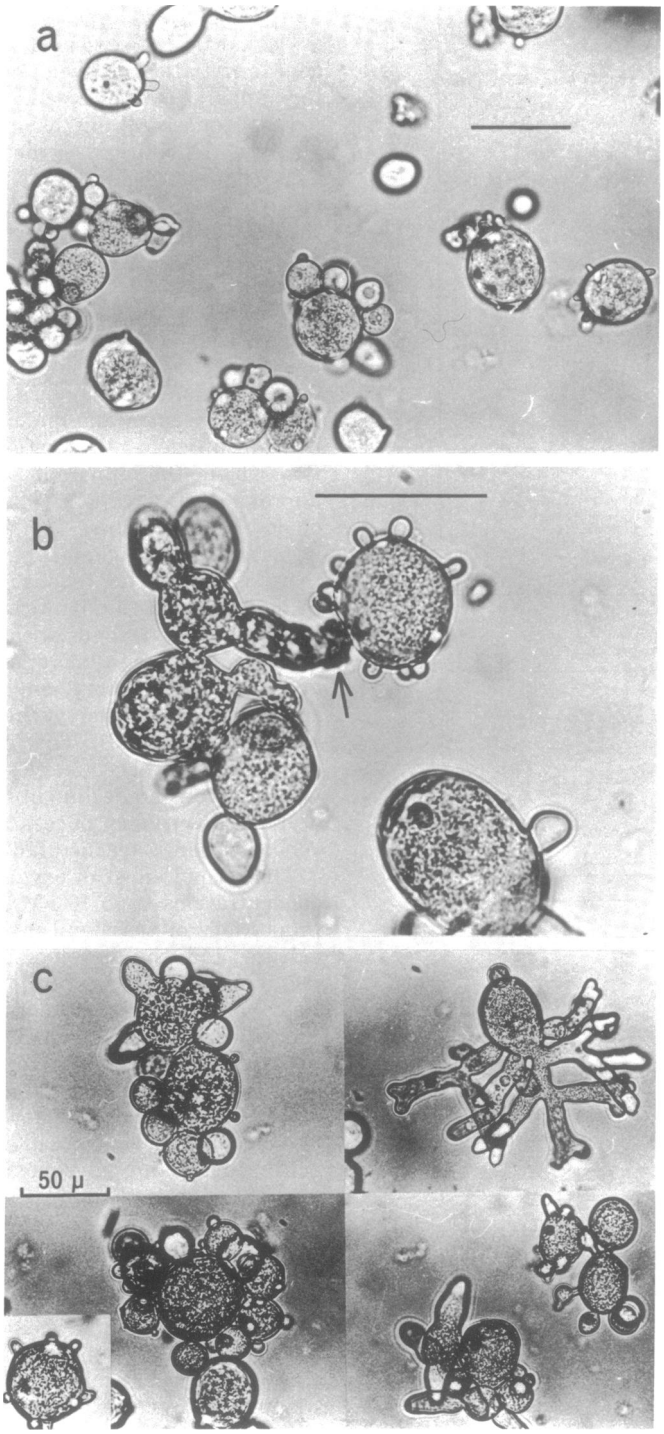

FIG. 7. *Photomicrograph of M. genevensis grown at high glucose concentrations (105 mM) and at a dissolved oxygen concentration of 15 μM. (a) and (b) illustrate the typical yeast-like morphology of these cultures, and the arrow in (b) indicates a cell bursting. (c) shows cells bearing stunted mycelial projections. The bar represents 50 nm.*

TABLE 3. *Morphological reversibility: the effects of anaerobiosis, glucose concentration, and chloramphenicol on the metabolism and morphology of established mycelial cultures*[a]

Steady state conditions		Yield (mg dry wt per mmol of glucose)	b_e (moles of ethanol per mole of glucose)	Respiration (ml of gas per g dry wt per h)			Yeast/yeast + mycelia (%)[b]
Oxygen (μM)	Glucose (mM)			Potential	Q_{O_2}	Q_{CO_2}	
A. 0.1	<0.025	51	1.8	15	<2	120	32
B. 70	220	40	1.5	17	5	110	98
C. 70 + chloramphenicol (12 mM)	<0.025	58	1.6	10	5	95	9

[a] The mycelial cultures were first established by growing *M. genevensis* under aerobic conditions (25 μM oxygen) in glucose-limited cultures before imposing the conditions described in this table. The input nutrient contained 55 mM glucose (A and C) and 330 mM glucose (B).

[b] The dimorphic ratio is expressed as the percentage by weight of the yeast phase compared to the sum of yeast and mycelial phases.

the concentration of these and other factors may occur during the transition to oxidative growth, and may contribute to the dimorphic response.

The results obtained from glucose-limited cultures provide a measure of the oxygen threshold for initiation of respiratory development, oxidative growth, and mycelial formation. However, if glucose is not the rate-limiting nutrient, but is present in excess, the morphology change can be suppressed. As oxygen concentration is increased in excess glucose cultures, the potential respiration increases and reaches a maximum of approximately 35 ml of oxygen per g dry weight per h (cf. 66 under glucose-limited conditions), but the actual respiration rate is very low (5 units, cf. 68 under glucose limitation), leading to incomplete metabolism of ethanol (Fig. 6). Thus, at higher oxygen concentrations, although the cell yield increases, there is little change from a fermentative mode of metabolism. These results indicate that mycelial development is not related to the formation of mitochondrial respiratory enzymes per se but may be determined by the development of a functional oxidative mode in the cell as a whole. Alternatively, yeast-like development may be unrelated to oxidative metabolism, being determined by other biochemical events regulated by glucose and other metabolites. The latter alternative seems more likely, in view of the fact that under glucose-limited conditions chloramphenicol inhibits the development of functional oxidative capacity in the cells, but does not induce yeast-like morphology in continuous culture. The fact that yeast-like forms are promoted by chloramphenicol in batch cultures (10, 28) is most probably due to the presence of higher glucose concentrations for most of the growth cycle.

After prolonged growth in aerobic conditions with glucose repression, some abnormal cells showing stunted hyphae appear, and lysis of some cells occurs. To account for this lysis we have considered the results of Bartnicki-Garcia (3), who has recently reviewed hyphal morphogenesis in fungi and has proposed a model to account for apical growth. Wall-lysing enzymes may be released from vesicles, which are abundant at the tips of growing hyphae, to loosen the structure of the cell wall, and to enable subsequent extension of the wall under the direction of specific synthetases. The autolysis observed in cultures grown at high glucose concentrations may be caused by an excessive accumulation of lytic enzymes, or by inhibition of the counteracting process of wall synthesis. Ethanol accumulation in the medium may be a cause of this cell lysis, since Bartnicki-Garcia (3) has found that dilute ethanol solutions can cause bursting of hyphae in *M. rouxii* and has suggested that this is caused by the release of lytic enzymes in apical vesicles.

The second objective of this study was to determine the morphological reversibility of highly aerobic mycelial cultures under conditions which would favor fermentative growth. It should be noted that yeast phase cultures grown from spores were always used to initiate the continuous cultures. Development, therefore, was always in the same direction: towards the mycelial phase characteristic of more aerobic conditions. When cultures were grown aerobically from spores, the resulting mycelial cultures failed to revert completely to yeast-like cultures when anaerobic conditions were imposed. Some formation of yeast-like cells was evident, but the cultures consisted predominantly of branched mycelia even after 20 generations under anaerobic conditions. Similarly, chloramphenicol had no effect on the morphol-

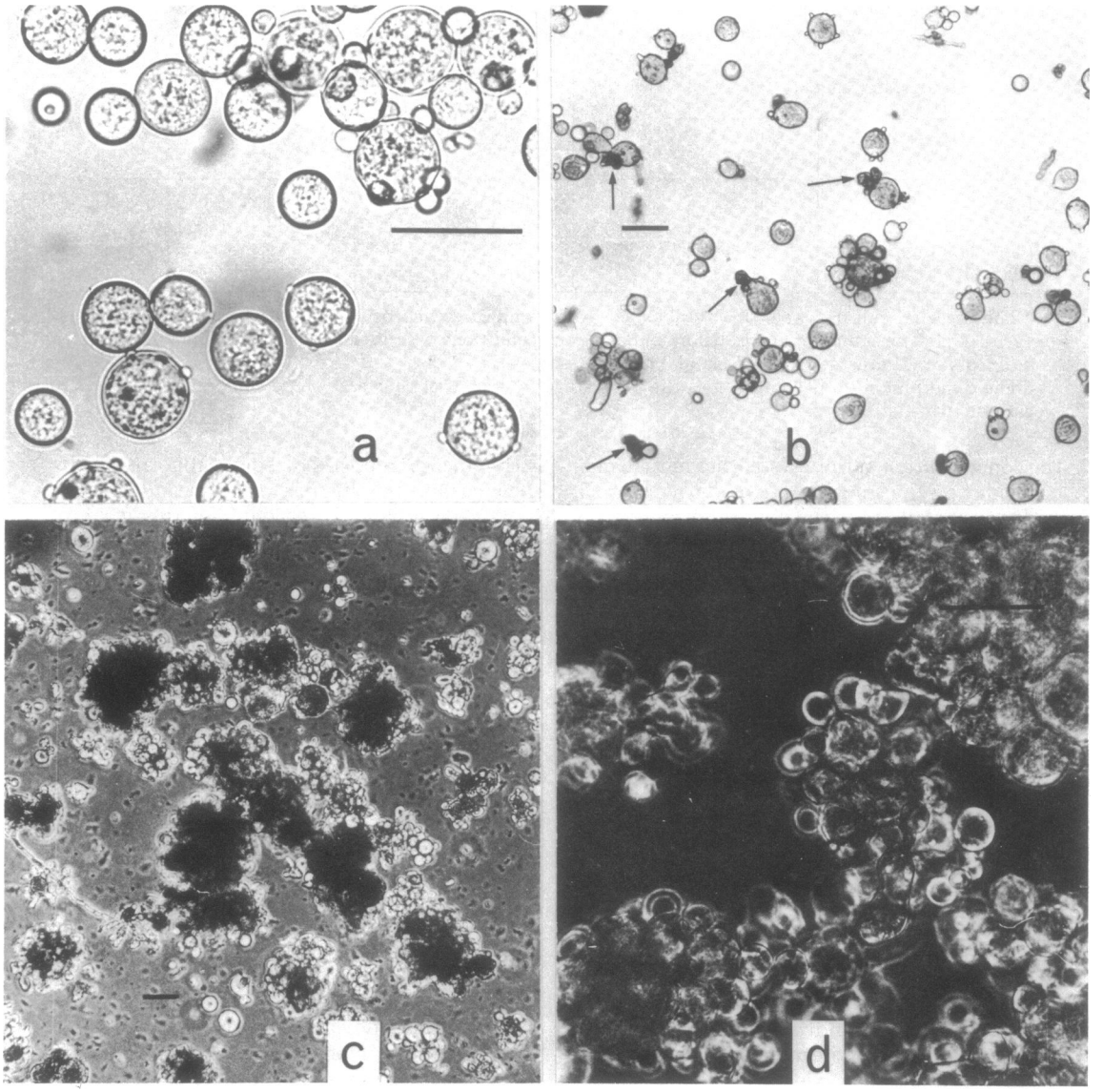

FIG. 8. *The effect of excess glucose on the morphology of aerobic cultures (15 μM) of M. genevensis. (a) ana (b) show cultures grown initially from spores and then continuously at high glucose steady states. (c) and (d) are micrographs of cultures obtained by subjecting previously glucose-limited aerobic mycelial cultures to high glucose concentrations (120 mM). The arrows in (b) indicate cell rupture similar to the sort noted in Fig. 7 (b).*

ogy of aerobically "committed" mycelial cells (Table 3) even though mitochondrial function is eliminated under these conditions. The metabolism in each of these cases as judged by the respiratory quotient and ethanol concentration in the medium was fermentative. However, high glucose concentrations, in addition to inhibiting oxidative growth, caused morphological reversion to yeast-like cells, which had a strong tendency to form clumps. We conclude from these observations that glucose plays a major role in determining the morphological status of *M. genevensis*.

The mechanism by which glucose may regulate morphogenesis in *Mucor* is not immediately evident. Although glucose does not repress mitochondrial development (as shown by measurement of potential respiration) in this organism, it does affect the oxidative capacity (actual respiration, and ethanol and carbon

dioxide production) of the cells. To this extent, glucose may thus cause general yet subtle metabolic shifts similar to those proposed by Wright (27) as underlying differentiation in the slime molds. Other possibilities which should also be considered include: effects on the concentration of key regulator molecules such as cyclic 3',5'-adenosine monophosphate (25); changes in the relative proportions and content of reduced adenine nucleotides (8); and differential effects on the activities of different pathways generating the building blocks for cell-wall synthesis.

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