NUTRITION, GROWTH, AND MORPHOGENESIS OF *MUCOR ROUXII*

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ABSTRACT

BARTNICKI-GARCIA, S. (Rutgers, The State University, New Brunswick, N.J.) AND WALTER J. NICKERSON. Nutrition, growth, and morphogenesis of *Mucor rouxii*. J. Bacteriol. **84**:841–858. 1962.—*Mucor rouxii* was grown under three different atmospheres of incubation: air, N_2, and CO_2 in parallel cultures. The atmosphere of incubation markedly affected nutritional requirements, growth, and morphogenesis. Absence of oxygen greatly reduced growth and increased the nutritional demands of the fungus. Presence of a high tension of CO_2 resulted in a change from filamentous to yeastlike morphogenesis. Aerobically, a large variety of carbon sources was utilized; anaerobically, only hexoses served to meet requirements for carbon and energy. Aerobically, various amino acids supported abundant growth; anaerobically, they were poorly utilized. Ammonium and nitrate ions were better sources of nitrogen for anaerobic growth. In general, incubation under either air or N_2 resulted in development of coenocytic filamentous mycelium, whereas incubation under CO_2 resulted in development of budding yeastlike cells. Variations in temperature and time of incubation, inoculum size, type and concentration of carbon source, type of nitrogen source, and presence of various substances with known action on fungal morphogenesis altered growth in many cases, but did not significantly affect the patterns of vegetative morphogenesis conditioned by each atmosphere of incubation. However, vegetative morphogenesis was strongly affected by addition of certain chelating agents. Yeastlike development of *M. rouxii* was prevented by ethylenediaminetetraacetic acid (EDTA) in concentrations which were also partially inhibitory for growth; under these conditions, development was filamentous. Chemically related chelating agents were similarly active. The growth-inhibitory and morphogenetic effects of EDTA were reversed by transition-group metal ions. Yeastlike development of *M. subtilissimus*, which does not require CO_2 for its induction, was also inhibited by EDTA.

The accompanying paper describes the controlling role of the atmosphere of incubation on mold-yeast dimorphism of *Mucor rouxii* (Bartnicki-Garcia and Nickerson, 1962*b*). Moldlike development resulted from incubation under either aerobic or anaerobic atmospheres containing relatively low tensions of CO_2. Yeastlike development occurred upon anaerobic incubation in the presence of relatively high tensions of CO_2. In the study reported herein, the constancy of the atmospheric control of morphogenesis was examined under a wide variety of environmental situations, including: modifications in time and temperature of incubation, size of inoculum, pH of the medium, type and quantity of carbon source, type of nitrogen source, presence of substances with known action of fungal morphogenesis, and addition of chelating agents. Simultaneously, determinations of total growth were made, thus extending the study to encompass a quantitative evaluation of the effect of atmosphere of incubation on growth and nutrition of *M. rouxii*.

MATERIALS AND METHODS

M. rouxii strain IM 80, of the culture collection of the Institute of Microbiology, and *M. subtilissimus* NRRL 1909, obtained through the courtesy of C. W. Hesseltine, Northern Utilization Research and Development Division, Peoria, Ill., were employed.

A defined medium was prepared with the following ingredients dissolved in distilled water to make 1 liter: glucose, 20 g; KH_2PO_4, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $ZnSO_4 \cdot 7H_2O$, 1.8 mg; $FeSO_4 \cdot 7H_2O$, 1.0 mg; $MnSO_4 \cdot H_2O$, 0.3 mg; $CuSO_4 \cdot 5H_2O$, 0.4 mg; thiamine, 1 mg; and nicotinic acid, 1 mg; pH was adjusted to 4.5 with

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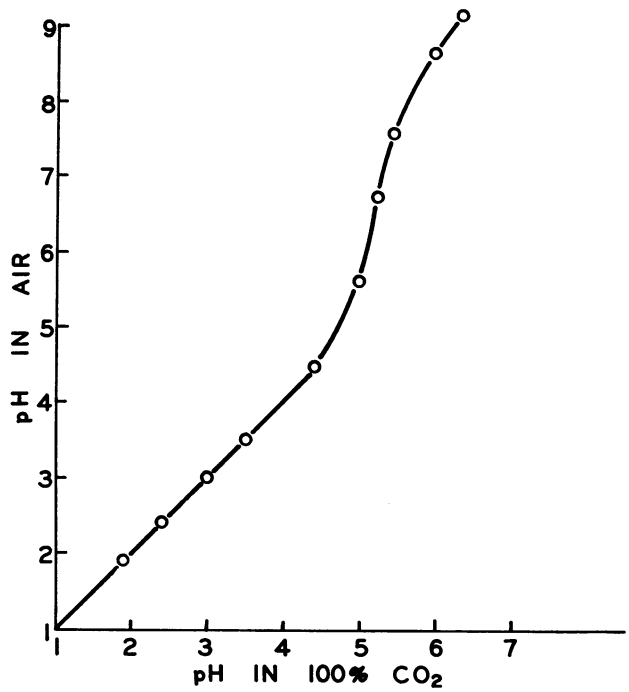


FIG. 1. *Modification of pH in YPG medium, adjusted to various pH values under air, after saturation with an atmosphere of CO_2 .*

dilute H_2SO_4 and the medium autoclaved at 121 C for 10 min. Thiamine and nicotinic acid (sterilized by filtration) and glucose (sterilized by autoclaving) were added aseptically to the basal salts medium. Nitrogen sources were added to the basal medium at a level of 0.5 g of N per liter. Each source was dissolved in water, adjusted to pH 4.5 with H_2SO_4 or KOH, and sterilized by filtration through a Millipore filter.

A complex liquid medium (YPG) composed of yeast extract, peptone, and glucose was prepared as previously described (Bartnicki-Garcia and Nickerson, 1962*b*). In some experiments, glucose in YPG medium was replaced by various carbon sources supplied at a level of 8 g of C per liter. Carbon compounds were dissolved in distilled water (organic acids were adjusted to pH 4.5 with KOH) and sterilized at 110 C for 10 min. They were then added to autoclaved yeast extract-peptone ingredients of YPG medium. Ethanol was added without sterilization. For experiments on the effect of pH on growth and form development, series of flasks in the range pH 1.9 to 9.1 were prepared by addition to autoclaved YPG medium of predetermined amounts of dilute H_2SO_4 or dilute K_2CO_3 to cover, respectively, values below and above pH 6.7, the value obtained on autoclaving YPG medium. Initial pH of each series was confirmed in control flasks equilibrated under air. These values were applied to cultures incubated under either air or N_2. For cultures incubated under CO_2 , initial pH was determined while the culture medium was maintained saturated with one atmosphere of CO_2. Figure 1 illustrates the change in pH of YPG medium upon saturation with CO_2. Increasingly greater acidification of the medium occurred above pH 4.5; below 4.5, saturation with CO_2 produced practically no change in pH. Final pH values were determined at the end of the incubation period under the same atmosphere used for determination of initial pH.

The following chelating agents, kindly donated by Geigy Industrial Chemicals, were employed: iminodiacetic acid (IDA), iminotriacetic acid (ITA), 1,2-diaminocyclohexane-*N*,*N'*-tetraacetic acid (CDTA), and diethylenetriaminepentaacetic acid (DTPA); the substances were suspended in water, and sufficient KOH was

added to dissolve them and to adjust pH to 4.5. Ethylenediaminetetraacetic acid (EDTA) in its disodium salt form ($Na_2EDTA \cdot 2H_2O$) was dissolved in water and pH adjusted to 4.5 with H_2SO_4. *o*-Phenanthroline (Phen) and 8-hydroxyquinoline (Oxine) were purchased from Matheson Coleman & Bell (East Rutherford, N.J.) and dissolved in 0.1 N HCl. Metal ion solutions were prepared by dissolving the following analytical reagent grade salts in distilled water: $CuSO_4 \cdot 5H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, $MnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 7H_2O$, $AlK(SO_4)_2 \cdot 12H_2O$, $MgSO_4 \cdot 7H_2O$, $CaSO_4 \cdot 2H_2O$, and $SrCl_2 \cdot 6H_2O$. Chelating agents and metals salts were sterilized by filtration through Millipore filters and added to autoclaved YPG medium. In all instances, final pH was 4.5.

All cultures were made in duplicate in 250-ml Erlenmeyer flasks containing 50 ml of culture medium. Flasks were agitated on a reciprocating shaker at 28 C for 48 hr. In all experiments, flasks were incubated under three different atmospheres: air, N_2, and CO_2. For incubation under air, flasks were simply covered with inverted 100-ml beakers; for incubation under N_2 or CO_2, a procedure described previously (Bartnicki-Garcia and Nickerson, 1962*b*) was employed.

Three types of inocula were used. (i) *Spores* (the preferred inoculum). The surface of solid YPG medium contained on the side of a 1,000-ml Blake bottle was inoculated with spores from a YPG slant. After incubation for about 1 week at 28 C, 10 to 20 ml of sterile distilled water were added, and the surface growth was rubbed gently with a sterile pipette. The heavy suspension of spores was centrifuged, washed three times with distilled water, resuspended in distilled water, and counted in a hemacytometer. Approximately 10^6 spores were routinely inoculated per 50 ml of culture medium. (ii) *Pregerminated spores.* Spores prepared as above were incubated in casein hydrolyzate-glucose medium supplemented with thiamine and nicotinic acid (Bartnicki-Garcia and Nickerson, 1961), under CO_2 for 8 to 12 hr. During this period, the spores germinated. They were harvested by centrifugation and washed three times with distilled water. About 10^6 pregerminated spores were inoculated per 50 ml of medium. (iii) *Yeastlike cells.* This inoculum, employed in experiments on effect of pH, was prepared by seeding spores of *M. rouxii* in liquid YPG medium and cultivating under CO_2 for 48 hr. Cells were harvested, centrifuged, and washed twice with distilled water. A volume containing approximately 3 mg (dry wt) of yeastlike cells was inoculated per flask.

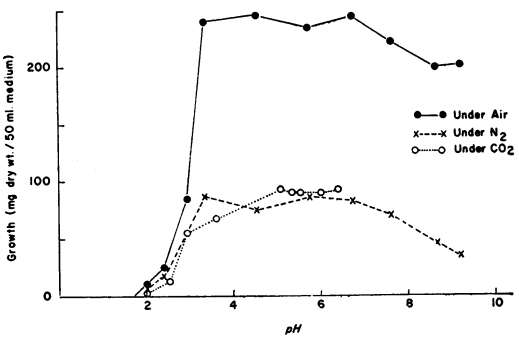


FIG. 2. *Effect of pH on the growth of Mucor rouxii incubated in liquid YPG medium under different atmospheres.*

In all experiments, growth was measured at the end of the incubation period by filtration of cultures through Pyrex fritted-glass crucibles (porosity M), followed by extensive washing with distilled water, drying at 80 to 90 C for 24 hr, and determination of dry weight.

RESULTS

Temperature of incubation. Mold-yeast dimorphism of certain fungi has been shown to be controlled solely by the temperature of incubation "thermal dimorphism" (Levine and Ordal, 1946; Nickerson and Edwards, 1949). Incubation of *Blastomyces dermatitidis* and *B. braziliensis* in the range of about 33 to 39 C determined yeastlike development; at lower temperatures, a filamentous growth developed. Shake cultures of *M. rouxii*, in YPG medium, incubated at 20, 28, and 37 C under the same atmosphere exhibited no change in morphological features, indicating that mold-yeast dimorphism in this fungus is temperature-independent.

Initial pH of culture medium. Under any of the three atmospheres of incubation (air, N_2, or CO_2) optimal growth of *M. rouxii* in YPG medium occurred over a wide range on the acid side, from approximately pH 3.5 to 7.0 (Fig. 2). Under CO_2, the most alkaline level that could be achieved was pH 6.3, which corresponded to pH 9.1 before the medium was saturated with CO_2 (Fig. 1). Aerobic cultures yielded crops at

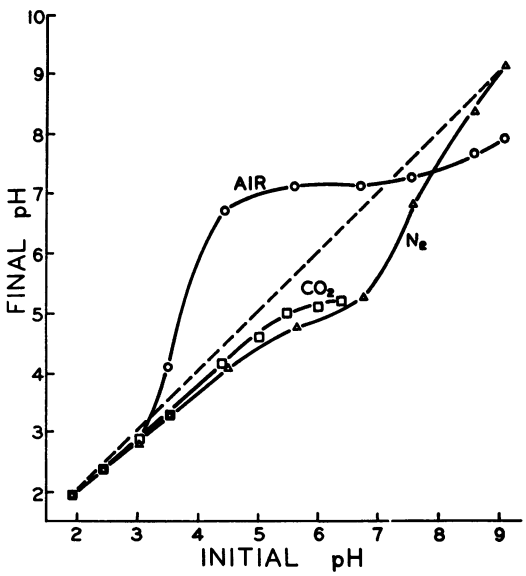


FIG. 3. *Final pH of liquid cultures of Mucor rouxii initially adjusted to different pH values. Cultures in YPG medium incubated for 48 hr under different atmospheres.*

the pH optimum that were three times greater than maximal crops obtained anaerobically. However, at levels more acid than pH 3, values for total growth were similar in cultures incubated aerobically or anaerobically.

The atmosphere of incubation influenced the final pH a culture attained. Anaerobically, there was a tendency to acidify the medium; aerobically, a tendency to neutralize the medium (Fig. 3).

Patterns of vegetative morphogenesis of *M. rouxii* were not altered upon cultivation at different pH values. Under aerobic conditions, the fungus developed a typically filamentous, abundant mycelium with a small proportion of fragmentary growth (*see* Bartnicki-Garcia and Nickerson, 1962*b*). At pH values more acid than 3.5, growth was sharply diminished; hyphae became shortened, with a distorted contour; cytoplasm was granulated and vacuolated; and round or ellipsoidal cells with thick distorted cell walls were observed. Cultures incubated under CO_2 displayed a purely yeastlike appearance; however, near neutrality (pH 6.0 and 6.3) a few cells developed short filaments. A clear effect of pH on morphology was observed in cultures incubated under N_2. There was a close relationship between pH and filamentation (Fig. 4). At pH 1.9, no growth occurred; only yeastlike cells from the inoculum were seen. At pH 2.4, the scant growth obtained consisted of swollen distorted cells with an ellipsoidal shape and fragile walls. At pH 3.0, growth was appreciable and composed of single cells with rudimentary branching. Some cells possessed a pyriform shape, possibly indicative of a preliminary stage in the formation of filaments. At pH 3.5, distinct filaments were seen, some with arthrosporal structures. As the pH of the medium was increased, the number and length of filaments increased accordingly. Above pH 4.5, practically all cells of the inoculum developed into filaments.

Inoculum size and incubation time. Preliminary experiments gave indication that size of inoculum affected form development in *M. rouxii*. Heavily inoculated cultures, incubated under N_2, exhibited a significant proportion of yeastlike cells amidst a typically filamentous growth. A detailed study was undertaken with three different concentrations of spores: (i) "heavy," 3.6×10^5 spores per ml of culture medium; (ii) "medium," 3.6×10^4 spores; and (iii) "light," 3.6×10^3 spores. Growth and morphology were recorded at 1, 2, 4, and 7 days in parallel flasks to evaluate their constancy over extended incubation periods.

In aerobic cultures, dissimilarities among growth curves resulting from the different inocula were minor and approximately proportional to size of inoculum (Fig. 5). Anaerobically, there were substantial differences between growth curves for filamentous and yeastlike forms. Yeastlike cells, growing under CO_2, attained essentially equal maximal growth, independent of inoculum size (Fig. 7); in contrast, under N_2, values for maximal growth of filamentous mycelium were smaller and dependent upon inoculum size (Fig. 6).

Inoculum size did not influence cellular form in cultures incubated under air or under CO_2; morphological features observed after 24 hr remained constant during the entire incubation period. In contrast, cultures incubated under N_2 showed drastically different morphological appearance, which was related to inoculum size (Fig. 8). Cultures which received a large inoculum exhibited an almost purely yeastlike appearance; most cells were spherical (a few with spherical buds), and only relatively few short filaments were seen. Distinctly filamentous growth was produced in cultures which received a medium

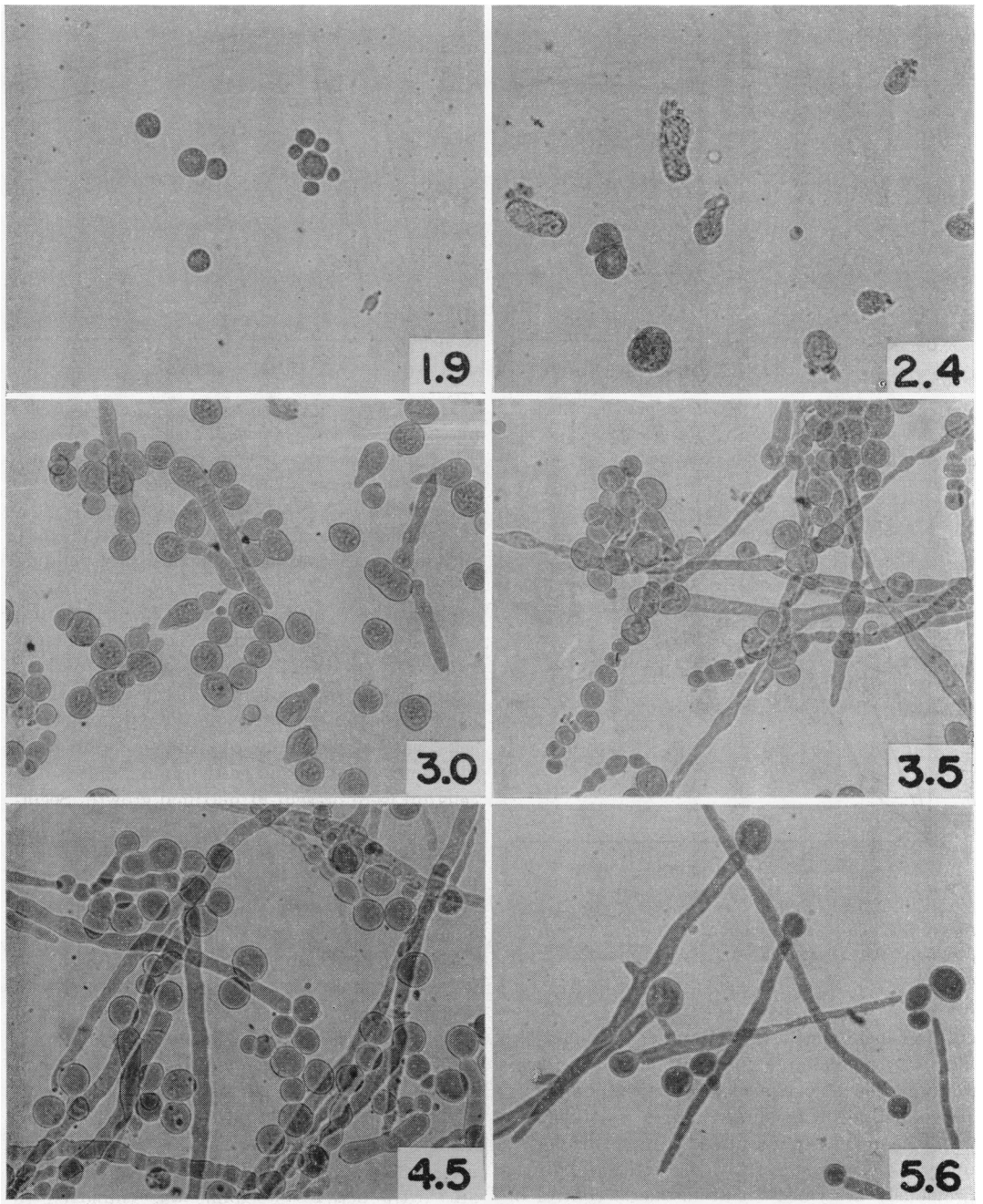


FIG. 4. *Effect of pH on morphology of Mucor rouxii grown under N_2 in YPG medium. Values for pH indicated on photographs; stain: lactophenol-cotton blue. Magnification: 225 ×.*

inoculum; filaments were short and without branches. With a light inoculum, unmistakable filamentous development of long hyphae was attained. This diversity of morphology was maintained during the entire incubation period, with only minor variations. Number and size of filaments in the most heavily inoculated cultures did not increase to any significant extent. In

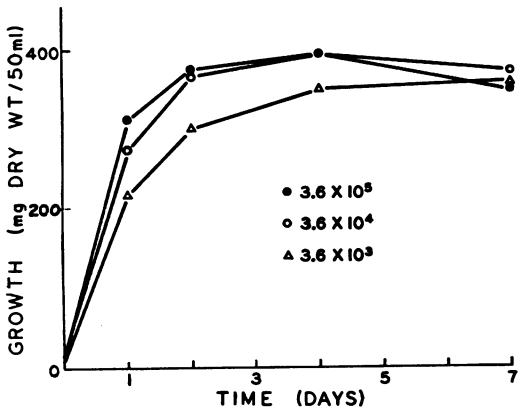


FIG. 5. *Effect of inoculum size on growth of Mucor rouxii incubated under air. Values given for the curves represent spores inoculated per ml.*

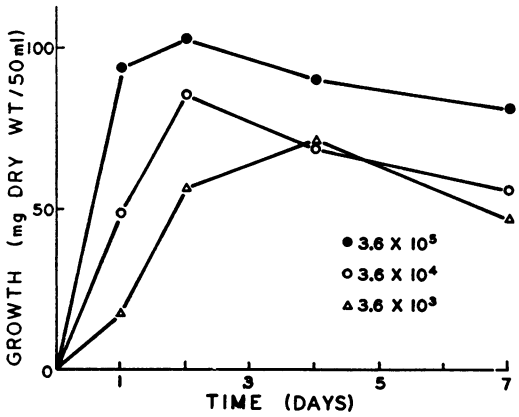


FIG. 6. *Effect of inoculum size on growth of cultures of Mucor rouxii incubated under N_2. Values given for the curves represent spores inoculated per ml.*

cultures incubated with medium or light inocula, filaments increased appreciably in length, and many of them developed into arthrospores.

Production of an almost purely yeastlike appearance in heavily inoculated cultures incubated under N_2 seems to be at variance with the findings previously described on atmospheric control of morphogenesis (Bartnicki-Garcia and Nickerson, 1962*b*). It may be argued, and perhaps with good reason, that a heavy inoculum would give rise to rapid utilization of glucose, with consequent increased evolution of CO_2; hence, ordinary flushing of cultures with N_2 would not suffice to remove metabolic CO_2 efficiently. While

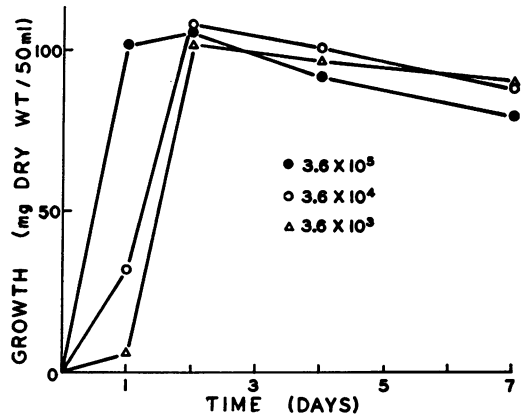


FIG. 7. *Effect of inoculum size on growth of cultures of Mucor rouxii incubated under CO_2. Values given for the curves represent spores inoculated per ml.*

this explanation is probably valid, and may account for the presence of some truly budding yeastlike cells, it is, however, only a partial explanation. A more important factor was uncovered when cell counts of the heavily inoculated cultures were made. (Due to absence of long filaments in such cultures, direct counts in a hemacytometer were feasible.) Total number of cells was estimated by counting buds or small filaments as individual cells. Approximately 10^6 cells/ml were present at maximal growth. Seemingly, all inoculated spores germinated; consequently, there was an average of one to two "cell divisions" to obtain maximal growth in cultures originally inoculated with 3×10^5 spores/ml. The tremendous increase in cellular dry weight (from 0.45 mg in the spore inoculum to 103 mg in a fully grown culture) was due mostly to weight augmentation of spores which enlarged from ellipsoids of $4.5 \times 5.5\ \mu$ to spheres of $18\ \mu$ average diameter. This increase in size corresponds to the preliminary stage of spore germination which leads to, but does not reach, filament formation. Further support for the above interpretation came from an experiment in which an "extra heavy" inoculum was employed (6×10^6 spores/ml). After 2 days of incubation under N_2, the resulting culture consisted entirely of spherical cells (9×10^6/ml) with no trace of filamentation; proliferation in this case was less than an average of one "cell division."

Glucose concentration. In liquid YPG medium, in which the initial concentration of glucose was

varied from 0 to 10%, growth of *M. rouxii* was directly proportional to glucose concentration in the range from 0 to 2% under either aerobic or anaerobic incubation (Fig. 9). At concentrations higher than 2%, the amount of growth varied according to the atmosphere of incubation. Under air, maximal growth was obtained with 5% glucose; higher concentrations were slightly detrimental, as measured after 2 days of incubation. Under N_2, the fungus exhibited maximal growth at 10% glucose, the highest concentration tested. Under CO_2, 2% glucose was optimal for growth.

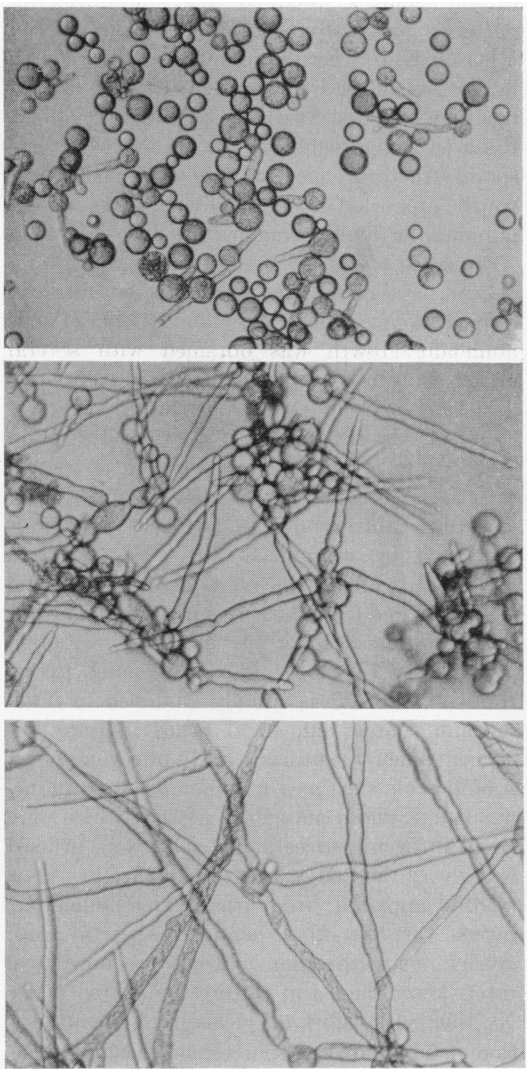


FIG. 8. *Effect of inoculum size on morphology of Mucor rouxii incubated in YPG medium under N_2 for 24 hr. Heavy inoculum, 3.6 × 10^5 spores/ml (top); medium inoculum, 3.6 × 10^4 spores/ml (middle); light inoculum, 3.6 × 10^3 spores/ml (bottom). Magnification: 225 ×.*

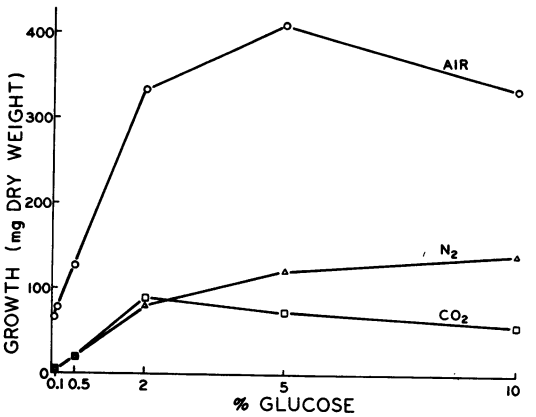


FIG. 9. *Effect of glucose concentration on growth of Mucor rouxii incubated for 48 hr in YPG medium under different atmospheres.*

It is noteworthy that in cultures incubated anaerobically, whether under CO_2 or N_2, there was absolutely no growth in the absence of glucose; there was not even swelling of spores that comprised the inoculum. On the other hand, when oxygen was available, significant mycelial growth occurred in media devoid of glucose. Evidently, yeast extract and peptone served to meet requirements for carbon and energy sources aerobically, but not anaerobically. Lack of spore germination in cultures incubated anaerobically without glucose suggested that glucose might be indispensable for spore germination and, thereafter, anaerobic growth might proceed without glucose. This possibility, however, was dispelled by failure of pregerminated spores to grow anaerobically in medium devoid of glucose.

Variations in glucose concentration did not significantly affect cellular form in cultures incubated under either air or CO_2. Cultures incubated under N_2, with less than 2% glucose, consisted entirely of coenocytic mycelium, whereas at higher concentrations the majority of filaments were septated and showed numerous arthrospores in various stages of development; isolated spherical cells were present, but multipolar budding was not observed (Fig. 10).

Carbon source. In view of the fact that *M. rouxii* was unable to utilize yeast extract and peptone as sources of carbon and energy in

cultures incubated anaerobically, glucose of YPG medium was replaced by different carbon compounds. Parallel cultures were incubated under air, N_2, or CO_2. Aerobically, the fungus utilized a wide variety of carbon compounds. Of the pentoses, xylose supported luxuriant growth, but growth with arabinose was little better than the control in basal medium. The four hexoses tested supported equally abundant growth, whereas, with disaccharides, maltose was readily utilized but sucrose and lactose were not (Table 1). In contrast, when the fungus was incubated anaerobically, only hexoses (glucose, mannose, fructose, or galactose) were utilized as sources of carbon and energy. Xylose and maltose, which supported the highest yields of mycelium under aerobic conditions, were totally inadequate to meet the carbon requirements of *M. rouxii* under anaerobic conditions.

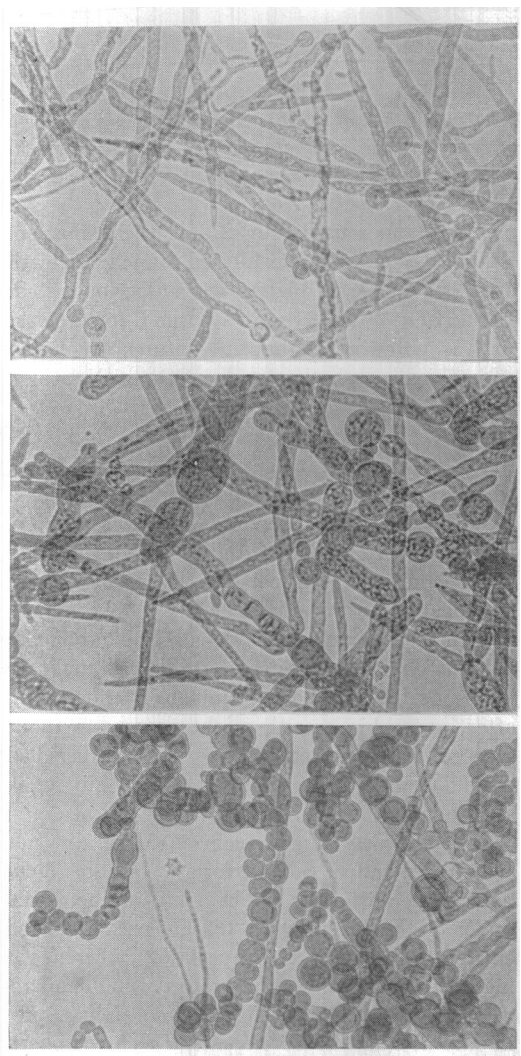


FIG. 10. *Effect of glucose concentration on morphology of Mucor rouxii grown under N_2 in YPG medium; 0.5% (top), 2.0% (middle), 5.0% (bottom). Stain: lactophenol-cotton blue. Magnification: 225 ×.*

In general, growth obtained with different carbon sources followed the morphological patterns previously described for growth in YPG medium. There were only slight variations in the extent of hyphal fragmentation of filamentous cultures. As a rule, the more abundant the growth the higher the proportion of arthrosporal structures; one exception was glucosamine, which supported good aerobic growth with a minimum of hyphal fragmentation.

Nitrogen source. M. rouxii was grown in a defined basal medium to which different nitrogen sources were added individually. Under air, abundant growth was obtained with several amino acids as sole nitrogen sources; L-glutamic acid and L-proline were particularly effective (Table 2). In the absence of oxygen, utilization of individual nitrogen sources was more restricted, especially in cultures incubated under CO_2. Anaerobic utilization of a nitrogen source was evaluated by comparison with its utilization aerobically. An aerobic-anaerobic growth ratio was calculated from the corresponding dry weight values obtained under the different incubation atmospheres (Table 3). It is to be noted that a ratio of 3 to 4 was usually obtained in YPG medium. Larger numerical ratios indicate less efficient anaerobic utilization of a nitrogen source. Amino acids such as L-glutamic acid, L-proline, DL-alanine, and γ-aminobutyric acid, which were good nitrogen sources aerobically, were utilized to a very limited extent anaerobically, as is readily apparent from their high numerical ratios. On the other hand, DL-aspartic acid, glycine, DL-methionine, and DL-threonine gave ratios lower than 5 in cultures incubated under N_2; thus, although total yields were low, utilization of these nitrogen sources, anaerobically, was in accord with the efficiency of their utilization aerobically. Incubation under CO_2 depressed, or virtually suppressed, amino acid utilization. On the basis of total growth, and aerobic-anaerobic growth ratios, only serine and threonine were significantly utilized. Anaerobic utilization of

TABLE 1. *Growth and form of Mucor rouxii cultivated with different carbon sources under various atmospheres**

Carbon source	Atmosphere					
	Air		N_2		CO_2	
	Growth	Form	Growth	Form	Growth	Form
D-Xylose	404	MF	0	IS	0	IS
Maltose	372	MF	0	IS	0	IS
D-Galactose	369	MF	79	MF	71	Y
D-Fructose	361	MF	66	MF	86	Y
D-Mannose	356	MF	67	MF	88	Y
D-Glucose	351	MF	82	MF	85	Y
2-D-Glucosamine·HCl	265	F	0	IS	0	IS
Succinic acid	181	MF	0	IS	0	IS
Ethanol	171	MF	0	IS	0	IS
Citric acid	138	MF	0	IS	0	IS
Fumaric acid	130	F	0	IS	0	IS
Adipic acid	128	F	0	IS	0	IS
Glycerol	127	F	0	IS	0	IS
D-Arabinose	112	MF	0	IS	0	IS
Starch	111	MF	0	IS	0	IS
Mannitol	110	F	0	IS	0	IS
Sucrose	106	MF	7	F	7	Y
Lactose	89	F	0	IS	0	IS
No addition	93	F	0	IS	0	IS

* Growth = mg (dry wt) per 50 ml of culture medium after 48 hr of incubation. Form: F = purely filamentous, MF = mostly filamentous with a few spherical cells, Y = purely yeastlike, IS = intact spores from inoculum.

TABLE 2. *Growth and form of Mucor rouxii cultivated with different nitrogen sources under various atmospheres**

Nitrogen source	Atmosphere					
	Air		Nitrogen		CO_2	
	Growth	Form	Growth	Form	Growth	Form
L-Glutamic acid	253	MF	12	MF	2	MY
L-Proline	222	MF	7	MF	4	Y
DL-Alanine	168	MF	7	FC	2	Y
γ-Aminobutyric acid	155	MF	1	MF	1	Y
L-Arginine·HCl	140	F	23	F	6	MY
Ammonium nitrate	120	MF	42	FY	31	MY
Urea	118	F	0	IS	0	IS
Ammonium sulfate	112	MF	27	MF	20	MY
DL-Methionine	111	MF	22	F	6	MY
Glycine	106	MF	27	F	4	YF
DL-Serine	100	MF	14	F	12	YF
Potassium nitrate	97	MF	29	MF	15	MY
DL-Phenylalanine	84	F	7	FC	8	MY
DL-Aspartic acid	58	MF	28	FY	5	MY
L-Histidine	44	F	1	F	3	Y
L-Lysine·HCl	16	F	2	F	2	Y
DL-Threonine	16	MF	10	F	4	YF
Cysteine·HCl	15	FC	0	IS	0	IS

* Growth = mg (dry wt) per 50 ml of culture medium after 48 hr of incubation. Form: F = purely filamentous, Y = purely yeastlike, MF = mostly filamentous with a few spherical cells, MY = mostly yeastlike with a few filaments, FY = filamentous with a higher proportion of spherical cells than MF, YF = yeastlike with a higher proportion of filaments than MY, FC = filamentous with numerous chains of arthrospores, IS = intact spores from inoculum.

TABLE 3. *Ratios of aerobic-anaerobic growth of Mucor rouxii cultivated in the presence of different nitrogen sources**

Nitrogen source	Atmosphere	
	Nitrogen	CO_2
L-Glutamic acid	21	127
L-Proline	32	56
DL-Alanine	24	84
γ-Aminobutyric acid	155	155
L-Arginine·HCl	6.2	23
Ammonium nitrate	2.9	3.9
Urea	—	—
Ammonium sulfate	4.15	5.6
DL-Methionine	5.0	19.5
Glycine	3.9	26.7
DL-Serine	7.1	8.3
Potassium nitrate	3.35	6.5
DL-Aspartic acid	2.1	11.6
L-Histidine	44	14.6
L-Lysine·HCl	8	8
DL-Threonine	1.6	4
Cysteine·HCl	—	—

* Ratios were calculated from data of Table 2. Ratio= dry wt. obtained under air divided by dry wt. obtained under anaerobic atmosphere.

inorganic nitrogen sources deserves special mention. Under either N_2 or CO_2 ammonium sulfate ammonium nitrate and potassium nitrate were better nitrogen sources than any of the amino acids tested.

Morphological characteristics of *M. rouxii* were affected differently by different nitrogen sources. Among the altered features were width, length, and uniformity of diameter of hyphae; extent of hyphal branching; presence, shape, and size of arthrospores; and size of yeastlike cells. Aerobically, cultures varied in macroscopic appearance from finely dispersed suspension of filaments to large clumps of filamentous growth. Microscopically, a typically filamentous morphology was seen, with modifications peculiar to each nitrogen source (Fig. 11 and 12). Hyphal width was highly variable, even in mycelium from the same flask. With ammonium nitrate, for example, hyphae ranged from 1 to 20 μ in diameter. Particularly thick hyphae were seen in cultures with ammonium sulfate, L-glutamic acid, and DL-aspartic acid. Cultures incubated under CO_2, wherein significant growth occurred, exhibited yeastlike development (Fig. 11) with a variable minor proportion of filamentous development. The proportion of filaments was especially high in cultures with DL-threonine, DL-serine, and glycine. With inorganic nitrogen sources, which supported the best growth under CO_2, a small proportion of filaments, some of considerable length, developed.

Cultures incubated under N_2 exhibited typically filamentous growth, with a variable proportion of arthrosporal structures. With L-arginine, DL-methionine, glycine, DL-serine, or DL-threonine, cultures were purely filamentous, whereas with ammonium nitrate or DL-aspartic acid, a large proportion of spherical arthrospores was seen amidst the filamentous growth. Numerous long chains of arthrospores developed with phenylalanine or DL-alanine as sole sources of nitrogen (Fig. 12).

Substances with reported action on fungal morphogenesis. Carboxylic acids have been reported to favor development of spherical cells in *Mucor* (Klebs, 1896; Ritter, 1913). According to Nadson and Philippov (1925), carbon dioxide could be replaced by tartaric acid as promoter of yeastlike growth of *M. guilliermondii*. The effect of lactic, pyruvic, oxalic, tartaric, oxaloacetic, malic, fumaric, succinic, α-ketoglutaric, glutaric, itaconic, and citric acids (all 0.1 M) on growth of *M. rouxii* was examined. The acids were dissolved in water, adjusted to pH 4.5, filter-sterilized, and added to autoclaved liquid YPG medium. Cultures were incubated under air, N_2, or CO_2. In no case was there evidence of replacement of the morphogenetic activity of CO_2 by a carboxylic acid.

Cysteine, which favors yeastlike growth of *Histoplasma capsulatum* (Salvin, 1949) and of *Candida albicans* (Nickerson and Van Rij, 1949), had no such effect on growth of *M. rouxii*, when tested at 6×10^{-3} M concentration in liquid YPG medium as described above.

Nickerson and Chung (1952) showed that cultures of *Saccharomyces cerevisiae* and *Zygosaccharomyces acidifaciens* grown in the presence of sodium fluoride (10^{-2} M) exhibited extensive clumping; buds did not abscise but remained attached to the mother cell. Growth of *M. rouxii* proved to be highly sensitive to NaF; complete inhibition of growth under air, N_2, or CO_2 was obtained at 2.5×10^{-3} M concentration, and only scant growth with 5×10^{-4} M NaF in YPG medium. Aerobically, a few typically multipolar budding yeastlike cells were formed

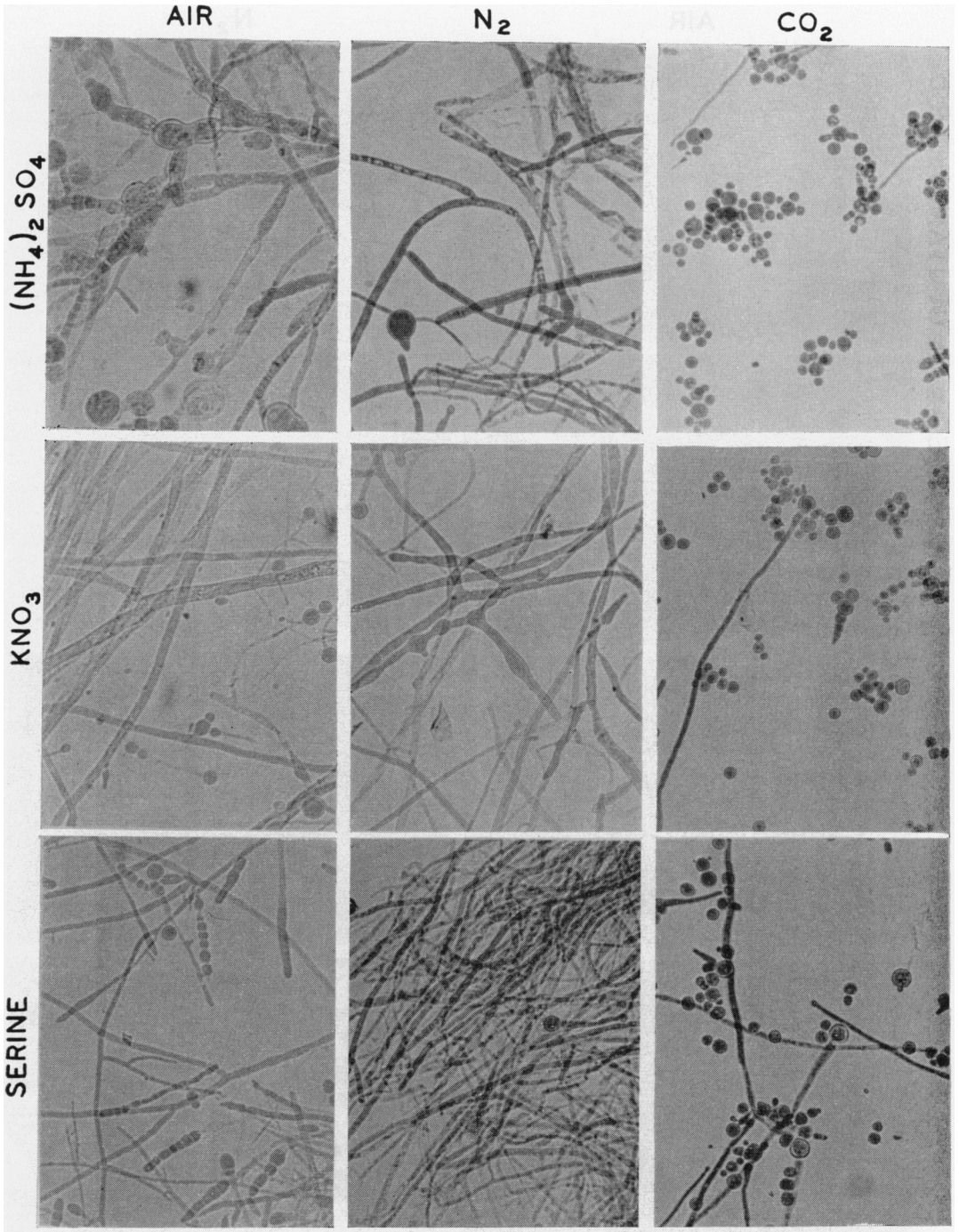


FIG. 11. *Morphology of cultures of Mucor rouxii incubated with three different nitrogen sources (horizontal rows) under three different atmospheres (vertical rows). Stain: lactophenol-cotton blue. Magnification: 225 ×.*

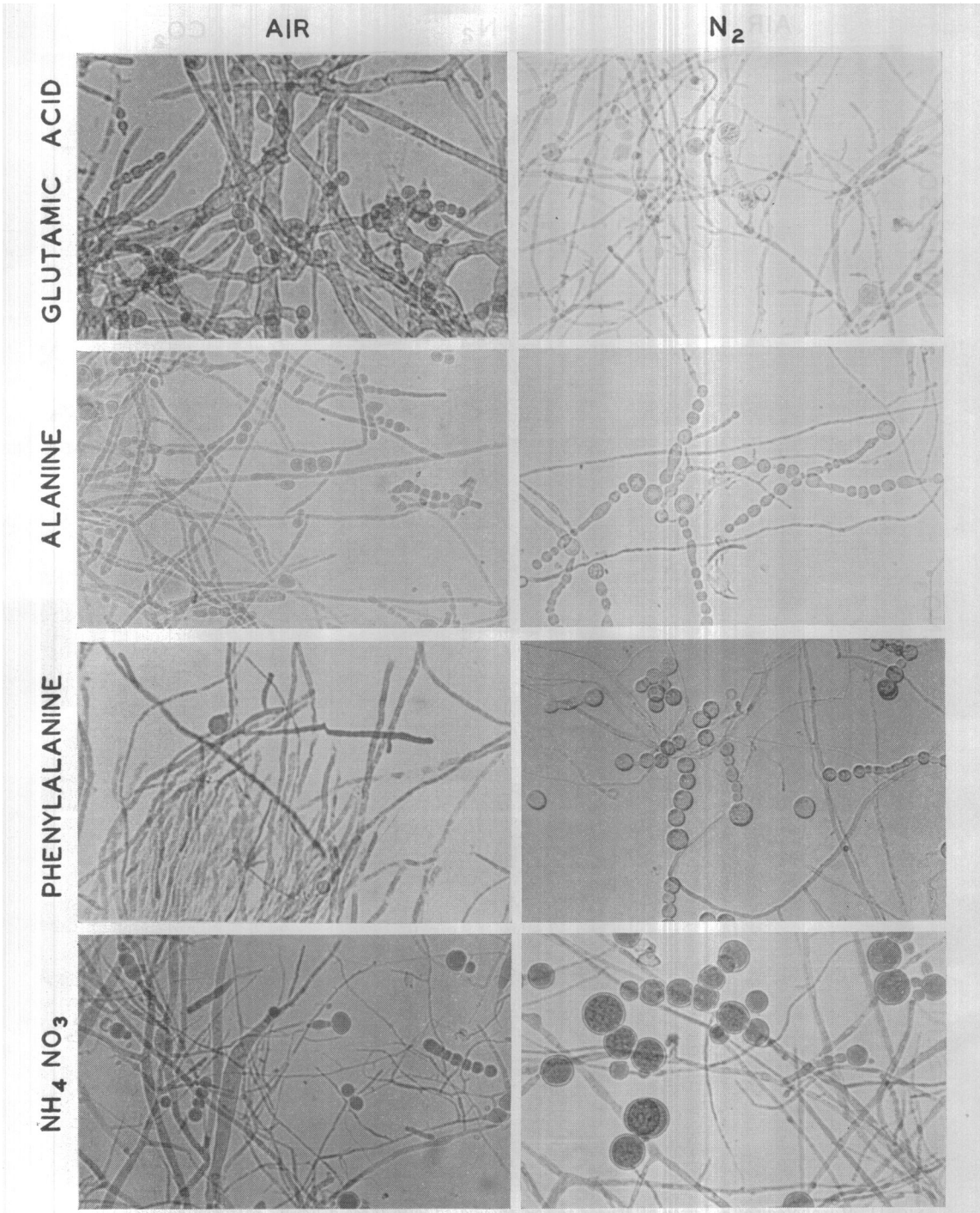


FIG. 12. *Morphology of cultures of Mucor rouxii incubated with various nitrogen sources under air or* N_2. *Stain: lactophenol-cotton blue. Magnification: 225* ×.

at the latter concentration. They appeared together with filamentous hyphae and other cellular structures consisting of densely granular ellipsoidal cells with multipolar spheroidal buds or with short hyphal tubes or both. The effect of NaF was also tested by adding several mg to the center of a YPG agar plate previously seeded on the surface with spores of *M. rouxii*. Duplicate sets of plates were incubated under air for 48 hr or under CO_2 for 96 hr. Plates incubated aerobically exhibited a central circle of inhibition surrounded by a ring of what appeared to be yeastlike colonies. Microscopically, the colonies were seen to consist of thick hyphae, with undulate contour and very short branches. Some of these branches resembled budding yeastlike cells. Plates incubated under CO_2 showed no effect other than a central zone of growth inhibition. The morphogenetic effect of NaF on *M. rouxii* mimics that obtained in *Neurospora crassa* incubated with high concentrations of sorbose (Terra and Tatum, 1961). Sorbose itself had no effect on *M. rouxii* when tested on solid medium as above. Under similar conditions, Tergitol 7 (sodium 3,9-diethyltridecanol-6-sulfate), a surface-tension depressor which affects morphology of *Neurospora* (Tatum, Barrat, and Cutter, 1949), had no effect on *M. rouxii*.

Inhibition of yeastlike morphogenesis by EDTA. In the presence of EDTA, cultures incubated under CO_2 were filamentous rather than yeastlike. The action of EDTA in nullifying the induction of yeastlike morphogenesis by CO_2 was examined in detail. Germinated spores of the fungus were inoculated into YPG medium containing various concentrations of EDTA, and cultures were incubated under air, N_2, or CO_2. In the range from 2.7×10^{-5} M to 2.7×10^{-4} M EDTA (0.001 to 0.01% Na_2 EDTA·H_2O), progressive inhibition of growth was observed under all atmospheres of incubation (Fig. 13). Concomitant with inhibition of growth, an increasing tendency toward filamentation was noted. This tendency was expressed in normally filamentous cultures (incubated under either air of N_2) by a decrease in the proportion of spherical cells (arthrospores), and by formation of longer and thinner filaments. In cultures incubated under CO_2, this tendency was dramatically manifested by a shift from yeastlike development to filamentous development. At 1.35×10^{-4} M EDTA, yeastlike morphogenesis was almost completely suppressed; growth was only one-third that obtained in the absence of EDTA, and consisted of long filaments with a few budding cells; some of the budding cells and buds were elongated. With higher concentrations of EDTA (2.7×10^{-4} M), the scant growth obtained was purely filamentous.

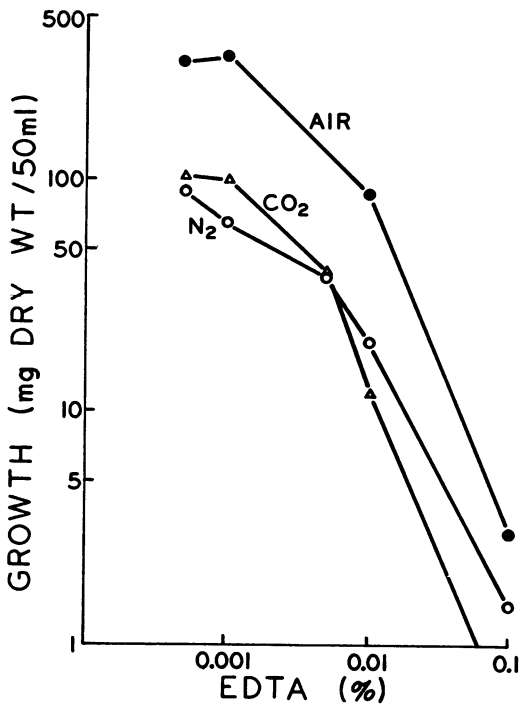


FIG. 13. *Effect of ethylenediaminetetraacetic acid on growth of Mucor rouxii incubated (48 hr) in YPG medium under three different atmospheres. Values given as per cent (w/v) of $Na_2EDTA \cdot 2H_2O$.*

Effect of other chelating agents on M. rouxii. IDA, ITA, CDTA, DTPA, Oxine, and Phen were added to YPG medium at various concentrations, and cultures were incubated for 48 hr under CO_2. All of these agents, except IDA, inhibited growth of *M. rouxii* (Fig. 14); DTPA and CDTA, which form the most stable metal complexes (Chaberek and Martell, 1959), were active at higher dilutions; IDA, with the lowest stability constants, showed no inhibitory action at the highest concentration tested (9×10^{-2} M).

Inhibition of growth by chelating agents of the *N*-acetic acid type (ITA, CDTA, DTPA) was accompanied by inhibition of yeastlike

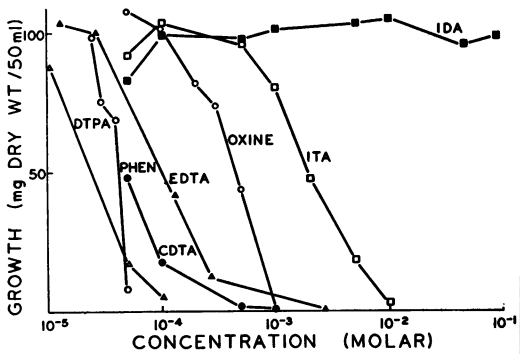


FIG. 14. *Effect of chelating agents on growth of Mucor rouxii incubated (48 hr) in YPG medium under CO_2. IDA = iminodiacetic acid; ITA = iminotriacetic acid; Oxine = 8-hydroxyquinoline; EDTA = ethylenediaminetetraacetic acid; CDTA = 1,2-diaminocyclohexane-N,N-tetraacetic acid; Phen = o-phenanthroline; DTPA = diethylenetriaminepentaacetic acid.*

morphogenesis and a shift toward filamentous development, as described above for EDTA. Fig. 15 illustrates the conversion from purely yeastlike development to purely filamentous development with increasing concentrations of DTPA.

Other chelating agents, not of the *N*-acetic acid type, namely, Phen and Oxine, did not significantly affect yeastlike morphogenesis of *M. rouxii*. Their growth inhibitory action was accompanied by only minor morphological modifications. Some pyriform buds appeared at highly inhibitory concentrations of Phen. Short, hook-shaped filaments developed from some spherical cells in the presence of inhibitory concentrations of Oxine.

Reversal of EDTA effect by metal ions. Metal ions at different concentrations were added to YPG medium containing 1.3×10^{-4} M EDTA; at this concentration, growth under CO_2 was inhibited to about one-fourth of the original value, and morphology was distinctly filamentous. Fig. 16 illustrates the antagonistic effect of metal ions on the growth inhibitory action of EDTA. Transition-group metal ions (Fe^{++}, Mn^{++}, Cu^{++}, Zn^{++}, Al^{+++}, or Co^{++}) at 10^{-4} M overcame EDTA inhibition of growth. With Co^{++}, however, growth was only partly restored; Zn^{++} ions were the most effective in reversing growth inhibition (3×10^{-5} M Zn^{++} sufficed to restore growth to normal levels). Higher concentrations of Zn^{++} were growth inhibitory. Alkaline earth metals (Mg^{++}, Ca^{++}, Sr^{++}), at the highest concentrations tested, did not significantly overcome growth inhibition.

The above mentioned cations also prompted reversal of the morphogenetic effect of EDTA, i.e., restoration of purely yeastlike development in cultures incubated under CO_2. The most active metals in this respect were Fe^{++}, Al^{+++},

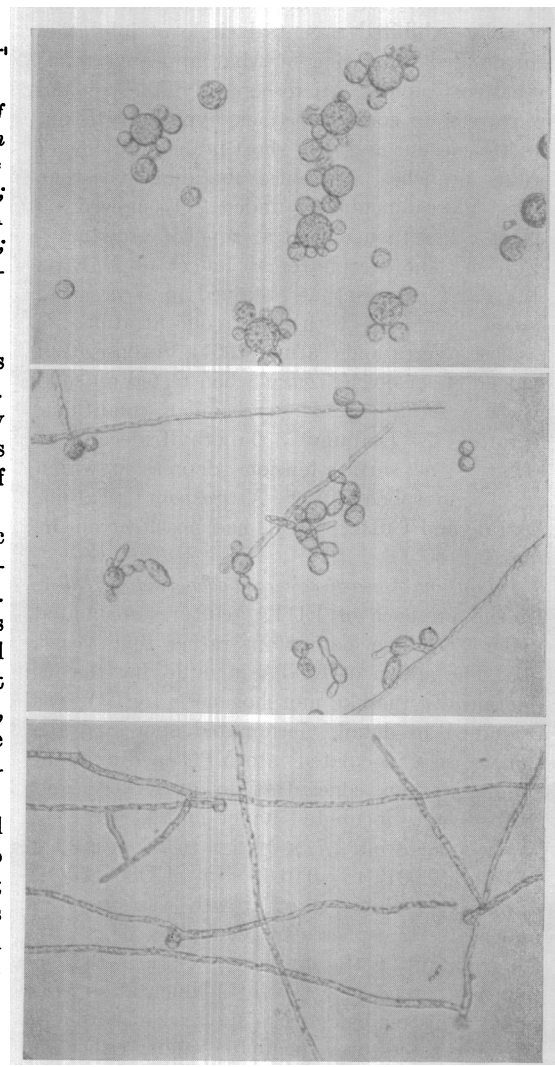


FIG. 15. *Inhibition of yeastlike morphogenesis of Mucor rouxii by diethylenetriaminepentaacetic acid (DTPA). Cultures incubated (48 hr) in YPG medium under CO_2 in presence of 10^{-5} M DTPA (top); 5×10^{-5} M DTPA (middle); and 10^{-4} M DTPA (bottom). Magnification: 225 ×.*

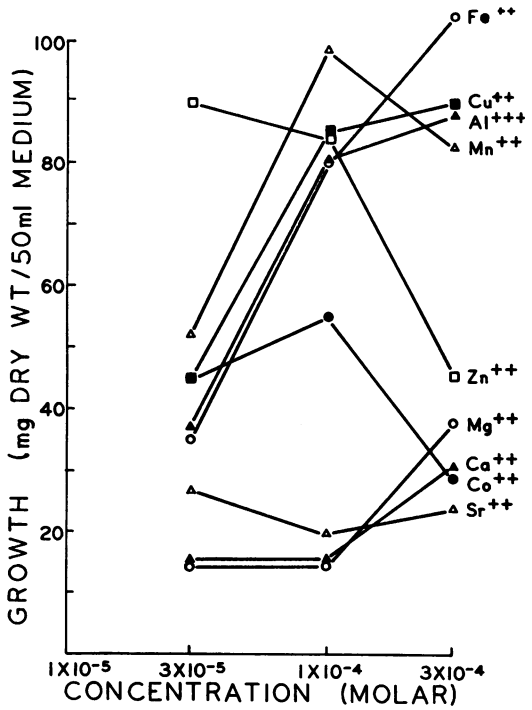


FIG. 16. *Reversal of EDTA inhibition of growth of Mucor rouxii by metals. Cultures incubated (48 hr) under CO_2 in presence of 1.3 × 10^{-4} M EDTA in YPG medium with concentration of cations as indicated.*

and Co^{++}. (At 10^{-4} M, a purely yeastlike develo p ment occurred in the presence of 1.3 × 10^{-4} M EDTA.) Mn^{++} and Cu^{++} were equally effective, but at a higher concentration (3 × 10^{-4} M). Alkaline earth metals, at the concentrations tested, were totally ineffective in overcoming the morphogenetic effect of EDTA. Zn^{++} deserves special mention; the growth inhibitory effect of EDTA was essentially overcome by 3 × 10^{-5} M Zn^{++} but the morphogenetic action was only partly reversed, and cultures showed mixed filamentous and yeastlike development.

Effect of EDTA on M. subtilissimus. A strain (NRRL 1909) of this fungus grows anaerobically in a purely yeastlike form without a requirement for CO_2 (Bartnicki-Garcia and Nickerson, 1962*b*). The morphogenetic effect of EDTA on the CO_2-independent yeastlike development of *M. subtilissimus* was compared with that observed in *M. rouxii*.

Spores of *M. subtilissimus* were inoculated into YPG medium, containing two different concentrations of EDTA, and incubated under N_2. A central well with 20% KOH solution was placed inside the flasks to minimize accumulation of metabolic CO_2. A growth inhibitory action of EDTA was clearly evident (Table 4). As with *M. rouxii* growth inhibition by EDTA was accompanied by a shift from yeastlike morphogenesis to filamentous morphogenesis.

TABLE 4. *Effect of EDTA on growth and morphogenesis of Mucor subtilissimus incubated in YPG medium under N_2*

EDTA	Growth	Morphology
M	*mg (dry wt)/50 ml*	
0	126	Purely yeastlike
0.5 × 10^{-4}	19	Mostly filamentous
1.3 × 10^{-4}	9	Purely filamentous

DISCUSSION

M. rouxii is capable of growing under a wide variety of environmental conditions. Its nutritional requirements are strongly conditioned by the presence or absence of oxygen in the atmosphere of incubation. Aerobically, the fungus can grow in simple defined medium with glucose as the only source of preformed organic matter. Anaerobically, nutritional requirements are more exacting with an absolute need for thiamine and nicotinic acid (Bartnicki-Garcia and Nickerson, 1961). In the presence of oxygen, the fungus can utilize a variety of carbon compounds, including alcohols, carboxylic acids, pentoses, hexoses, disaccharides, amino sugars, and amino acids. In the absence of oxygen, only hexoses serve as source of carbon and energy.

Failure of the fungus to utilize disaccharides (such as maltose) in the absence of oxygen poses a difficult problem of interpretation. Conceivably, failure of maltose to be hydrolyzed to glucose (readily utilized anaerobically) might account for lack of maltose utilization. However, as far as is known, no oxygen requirement is associated with enzymatic hydrolysis of maltose or any other oligosaccharide. Some yeasts manifest a similar behavior in their carbohydrate utilization. The older literature was reviewed by Kluyver and Custers (1940), who discussed possible interpretation of the situation wherein certain yeasts assimilate and oxidize disaccharides which they do not metabolize anaerobically. Recently, Harris

and Thompson (1961) obtained evidence indicating that penetration of maltose into cells of *Saccharomyces cerevisiae* is mediated by a specific permeation mechanism, vulnerable to metabolic poisons. Conceivably, penetration of maltose (and some other substrates) into *M. rouxii* may also occur through a specific permeation mechanism which, to account for our observations, might be inoperative in the absence of molecular oxygen.

A substantial amount of (HCO_3^- + $CO_3^=$), derived from the K_2CO_3 that was used to adjust pH, was present in cultures with an initially alkaline reaction. The combined amount of bicarbonate and carbonate present in anaerobic cultures (under N_2) at pH 9.1 was 0.025 M. In such cultures, there was no tendency toward yeastlike development; on the contrary, growth was markedly filamentous, indicating that bicarbonate or carbonate are not inducers of yeastlike morphogenesis. Most likely, molecular CO_2 is the active morphogenetic agent, as further evidenced by the fact that CO_2 displays full activity on morphogenesis at pH values below 4, where bicarbonate concentration is nil and essentially all of the carbon dioxide is present as physically dissolved CO_2.

Although the basic patterns of vegetative morphogenesis of *M. rouxii* were invariably determined by the composition of the atmosphere of incubation, other environmental factors exerted minor effects on morphogenesis, leading in some instances to concomitant development of filaments and yeastlike cells. Incubation anaerobically under CO_2 always resulted in yeastlike development but, depending on the nitrogen source, a variable proportion of filaments might also be formed. This effect was especially noticeable with glycine, DL-serine, and DL-threonine as sole sources of nitrogen. Abundant yeastlike development, completely devoid of filamentous growth, was only obtained with complex nitrogen sources such as yeast extract-peptone or vitamin-free casein hydrolysate (Bartnicki-Garcia and Nickerson, 1961), suggesting that a multiple amino acid diet may be necessary to exclude filamentation in cultures incubated under CO_2.

Cultures incubated in the absence of CO_2 were filamentous under the broad range of environmental conditions tested. Frequently, however, a variable but significant proportion of spherical cells was formed. These spherical cells were not yeastlike cells but arthrospores. As described earlier (Bartnicki-Garcia and Nickerson, 1962*b*), arthrospore formation is a normal event following filament development in *M. rouxii*.

Under certain cultural conditions, such as a very acidic medium, use of a heavy inoculum, or a high glucose concentration, cultures incubated anaerobically in the absence of CO_2 abounded in spherical cells that, superficially, gave the misleading impression of yeastlike development. The effect of an acidic environment favoring "yeastlike" development of *Mucor* has been claimed (Brefeld, 1873; Ritter, 1913, Nadson and Philippov, 1925). However, mycologists do not always make a clear distinction between arthrospores and yeastlike cells. Whereas the findings of Brefeld, and of Nadson and Philippov, actually seem to pertain to truly budding yeastlike cells, Ritter's report of the effect of an acidic environment on *M. spinosus* and *M. racemosus* was undoubtedly on promotion of arthrospore formation. Our results indicate that yeastlike development of *M. rouxii* is essentially independent of pH. Although numerous spherical or ellipsoidal cells were formed in anaerobic cultures more acid than pH 4, no evidence of budding was seen. Spherical cells which developed between pH 3 to 4 were derived by arthrospore formation, and in cultures with a more acid initial pH were derived from the swelling of cells contained in the inoculum. Similarly, enlargement and rounding out of spores gave rise to the misleading yeastlike appearance of anaerobic cultures (N_2) heavily inoculated with spores of *M. rouxii*.

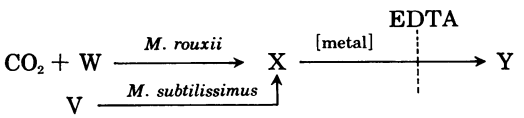
Our results indicate that a high sugar concentration is not necessary for, or stimulatory to, yeastlike development. Purely yeastlike growth was obtained with the lowest concentration of glucose tested (0.1%) if cultures were incubated under CO_2. The favorable action of a high concentration of sugar on spherical cell formation in *Mucor* reported by Klebs (1896) and Ritter (1913) would appear to be a stimulation of arthrospore formation. In fact, Ritter stated that his yeastlike cells (Mucorhefe) did not exhibit budding. Anaerobic cultures (N_2) of *M. rouxii* with increasing concentrations of glucose produced increasingly higher proportions of arthrospores.

It is important to emphasize that, although growth of yeastlike cells of *M. rouxii* occurs only in the presence of hexoses, these sugars have no effect per se on morphogenesis; they serve merely

to meet the nutritional demands imposed by the obligatory anaerobic life inherent to yeastlike development.

Susceptibility of vegetative morphogenesis of *Mucor* to control by chelating agents of the N-acetic acid type originates, most likely, from inactivation or depletion of a specific metal(s) required in metabolic reactions intimately associated with form development. Control of morphogenesis by chelating agents occurred with a concomitant inhibition of growth. The two actions, however, are not obligatorily associated. Thus, growth inhibition by EDTA may be completely reversed by relatively low concentrations of Zn^{++}, insufficient to restore a purely yeastlike development. Chelating agents not of the N-acetic acid type (Oxine, Phen) caused inhibition of growth without morphogenetic alterations.

In view of the existence of strains of *M. subtilissimus* which do not require CO_2 for induction of yeastlike morphogenesis (Bartnicki-Garcia and Nickerson, 1962*b*), the question arises as to whether these strains possess a basically different mechanism for induction of yeastlike morphogenesis, or whether an otherwise similar mechanism became carbon dioxide-independent. The latter possibility seems more likely since both oxygen and EDTA equally inhibit induction of yeastlike morphogenesis in carbon dioxide-dependent and carbon dioxide-independent strains of *Mucor*. One may, therefore, postulate a common internal inducer of yeastlike development, "Y", the formation of which in *M. rouxii* requires the presence of CO_2, but which can be synthesized anaerobically in the absence of CO_2 in *M. subtilissimus*. The situation may be visualized in the following manner:



where V, W, and X are metabolic precursors of Y.

Our findings indicate that EDTA inhibits a reaction common to both types of *Mucor*. It would, thus, appear that CO_2 is not the ultimate inducer of yeastlike development but part of a pathway leading to a common inducer of yeastlike morphogenesis whose formation requires the presence of some metal(s).

The cell wall being the structure ultimately responsible for cellular morphology of *M. rouxii*, the action of Y may be postulated to lead to an alteration of cell-wall polymers normally present in the filamentous form. This hypothesis has been substantiated by the finding of marked, and presumably morphogenetically significant, differences in chemical composition and structure of cell walls of the aerobic filamentous form and yeastlike form of *M. rouxii* (Bartnicki-Garcia and Nickerson, 1962*a*).

ACKNOWLEDGMENT

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