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Nutritional Requirements for Growth and Yeastlike Development of *Mucor rouxii* Under Carbon Dioxide

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Unlike five other strains of *Mucor rouxii* previously studied, certain nutritional factors must be present for rapid growth and completely yeastlike development of *M. rouxii* (National Regional Research Laboratory 1894) under CO_2; high CO_2 tensions markedly inhibit growth of this strain. Addition of yeast extract, peptone, or enzymatically hydrolyzed casein in substrate amounts to a basal medium (containing acid-hydrolyzed casein) completely relieved CO_2 inhibition of growth and permitted yeastlike development. The "CO_2 growth factor" activity of these supplements proved to be dialyzable and acid labile. These findings, together with the results of gel filtration and amino acid analysis, suggested that CO_2 growth factor activity can be attributed to small peptides.

Although a large number of fungi are capable of growing anaerobically, only a very few can grow under high pCO_2 atmospheres (18). Studies on the effect of high CO_2 and low O_2 tensions on soil microbiota have shown that many fungi from tropical soils were capable of developing under 100% N_2, but only a few fungi could grow under a CO_2 concentration greater than 95%, even in the presence of oxygen (16, 17). Of eight genera of *Mucorales* surveyed, only *Rhizopus* (5 of 6 species tested) and *Mucor* (11 of 12 species tested) grew under 100% CO_2 on a complex medium. (4).

CO_2 at pCO_2 values of 0.3 or higher, induced yeastlike development in five strains of *Mucor rouxii*, whereas growth under air or nitrogen was filamentous. An atmosphere of 100% CO_2 inhibited growth of these strains of *M. rouxii* only slightly (4). Similarly, the morphology of *M. rouxii* NRRL (National Regional Research Laboratory) 1894 under air or N_2 is filamentous, provided the inoculum size is not excessive (7). However, unlike the five other strains of *M. rouxii* previously studied, we have found that certain nutritional factors must be present (in addition to CO_2) for yeastlike development of this strain. Furthermore, high CO_2 tensions are growth inhibitory. Addition of supplements containing small peptides to a defined medium markedly stimulated growth and permitted yeastlike development of this strain of *M. rouxii* under 100% CO_2.

MATERIALS AND METHODS

Organism. *M. rouxii* NRRL 1894 was obtained through the courtesy of C. W. Hesseltine (Northern Utilization Research and Development Division, Peoria, Ill.).

Media. The basal medium consisted of a defined medium (5) to which acid-hydrolyzed casein (0.2% final concentration) was added. The YPG medium (4) consisted of the following, dissolved in distilled water to make 1 liter: yeast extract, 3 g; peptone, 10 g; and glucose, 20 g. All media were adjusted to *p*H 4.5 with dilute H_2SO_4. All amino acids and vitamin supplements were sterilized by filtration through filters (0.45 μm pore size; Millipore Corp., Bedford, Mass.) and added aseptically to the autoclaved media. The "strepogenin supplement," prepared as described by Kihara and Snell (9), contained the following per liter of final medium: L-ascorbic acid, 500 mg; L-cysteine, 400 mg; L-glutamine, 300 mg; L-serine, 100 mg; guanylic acid, 50 mg; uracil, 10 mg; and spermine·4 HCl, 1 mg.

Cultivation methods. The microbiological procedures and incubation apparatus employed were as previously described (7); in all instances, the inoculum size was 10^4 spores per ml. When indicated, a scaled-down version of the usual incubation system was employed as follows. All cultures were made in duplicate in cotton-plugged vials (23, inside diameter, by 62.5 mm high) containing 5 ml of medium. Vials containing media were sterilized for 6 min at 125 C and inoculated immediately when cool. The cultures were placed on a rack in a large covered jar (a desiccator, 260, inside diameter, by 300 mm high) containing the desired atmosphere of incubation. The lid was fitted with a gas inlet tube that reached to the bottom of the

jar and an outlet tube connected to a water seal. The desired atmosphere was maintained by continuously flushing the system with the appropriate water-saturated gas mixture. The covered jar was attached to a rotary shaker, and cultures were incubated at 28 C for 3 days at 150 rev/min.

Growth measurements. The amount of growth (dry weight) was determined as previously described (5). When the scaled-down incubation system was used, growth was measured as follows. The entire 3-day-old culture was homogenized in a Ten-Broeck tissue homogenizer, and the cells were separated by centrifugation and suspended in an appropriate volume of distilled water. Turbidity measurements (420 nm) were correlated to dry-weight values by a standard curve. Most cultures that developed in a filamentous manner grew very slowly, and growth at 3 days consisted of swollen spores with protruding short germ tubes. Turbidity data on these slow-growing filamentous cultures corresponded well with actual dry-weight measurements. When filamentous growth was extensive, dry-weight determinations were made directly by drying washed cells at 90 C for 24 hr in tared aluminum pans.

Lipid extraction. A procedure based on the method of Anderson, as described by Peck (12), was employed as follows: 10 g of peptone was extracted with 200 ml of 95% ethyl alcohol-ethyl ether (1:1) for 14 hr and with 200 ml of chloroform for 4 hr in a Soxhlet extraction apparatus. Some of the residue (200 mg) was then extracted with 200 ml of 95% ethyl alcohol-ethyl ether (1:1) containing 2.0 ml of 12 N HCl for 5 hr at 50 C. The residue from this treatment was extracted with 95% ethyl alcohol-ethyl ether (1:1) for 12 hr and with chloroform for 8 hr in the Soxhlet apparatus.

Ashing procedure. Peptone was ashed by heating at 600 C for 6 hr in a muffle furnace.

Dialysis. A 5% solution (100 ml) of peptone or yeast extract or 100 ml of a 2.5% solution of enzymatically hydrolyzed casein was dialyzed against 1 liter of distilled water at 2 C for 36 hr with two changes of water at 12-hr intervals.

Gel filtration. Peptone dialysate, concentrated in vacuo, was fractionated on a column (21 by 700 mm) of Sephadex G-10 (100 g) suspended in 0.025 M phosphate buffer (*p*H 7.0). The dialysate material (630 mg in 4 ml of the above buffer) was applied to the column and eluted with the phosphate buffer at a rate of 0.5 ml per min. The temperature was maintained at 2 C. Blue dextran 2000 (Pharmacia, Inc., Piscataway N.J.), which is completely excluded from the gel matrix, was used to determine the void volume (V_0). In addition to the V_0 (90 ml), 10 fractions of 12 ml each were collected. The V_0 contained 279 of the 630 mg of dialysate added to the column.

Analytical procedures. Amino acids were analyzed by using a Technicon amino acid analyzer. Preparations were hydrolyzed with 6 N HCl (1 ml per 5 mg of sample) under N_2 in a sealed tube at 103 C for 24 hr, unless otherwise indicated. Acid was removed in vacuo during agitation on a Buchler Evapomix.

Chemicals. Carbon dioxide (bone dry grade, 99.8% purity), nitrogen (prepurified grade, 99.997% purity), and 30% carbon dioxide (balance N_2) were purchased from the Matheson Co., Inc., East Rutherford, N.J. Yeast extract, peptone, and yeast nitrogen base were purchased from Difco. Enzymatically hydrolyzed casein and acid-hydrolyzed casein (salt free, vitamin free) were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

RESULTS

Effects of pCO_2 and medium on growth and morphology. The pCO_2 of the incubation atmosphere determines the morphology of growth on the complex medium (Fig. 1A–C). Completely yeastlike growth was obtained under 100% CO_2. Growth on the basal medium was mostly filamentous under all atmospheres tested (Fig. 1D–F).

The medium and the atmosphere of incubation also affect the growth rate and the total amount of growth (Fig. 2). On the YPG medium, growth was stimulated by CO_2 and most growth was obtained under 100% CO_2. In contrast, on the basal medium a 100% CO_2 atmosphere inhibited growth. Whereas growth was substantial after incubation for 58 hr under 30% CO_2 (1.6 g per liter) and N_2 (1.1 g per liter), growth under 100% CO_2 was only about 0.2 g per liter.

Supplementation of basal medium. It would appear that the complex medium contained some factor(s) that mitigated the inhibitory effect of high CO_2 tensions and, together with CO_2, permitted yeastlike development. In an attempt to define the nature of the factor(s), various components of the YPG medium and enzymatically hydrolyzed casein were added to the basal medium to test their effect on growth and morphology under 100% CO_2 (Fig. 3). The results show that the basal medium, when supplemented with either yeast extract, peptone, or enzymatically hydrolyzed casein, supported rapid growth under CO_2. Growth in the supplemented media was yeastlike, whereas the slight amount of growth in the basal medium was mostly filamentous. Further experiments showed that a medium in which the acid-hydrolyzed casein in the basal medium was replaced by any of the above three supplements supported rapid growth and a predominantly yeastlike development. Increasing the content of acid-hydrolyzed casein in the basal medium had no effect on growth or morphology.

The activity of peptone does not appear to be due to the presence of contaminating lipids or inorganic substances since (i) lipid-extracted peptone retained most of the CO_2 growth factor activity, (ii) lipid-extracted peptone was active in supporting yeastlike development ("Y-factor" activity), and (iii) addition of peptone ash to the basal medium had no effect on growth or development.

Peptone, yeast extract, and enzymatically hy-

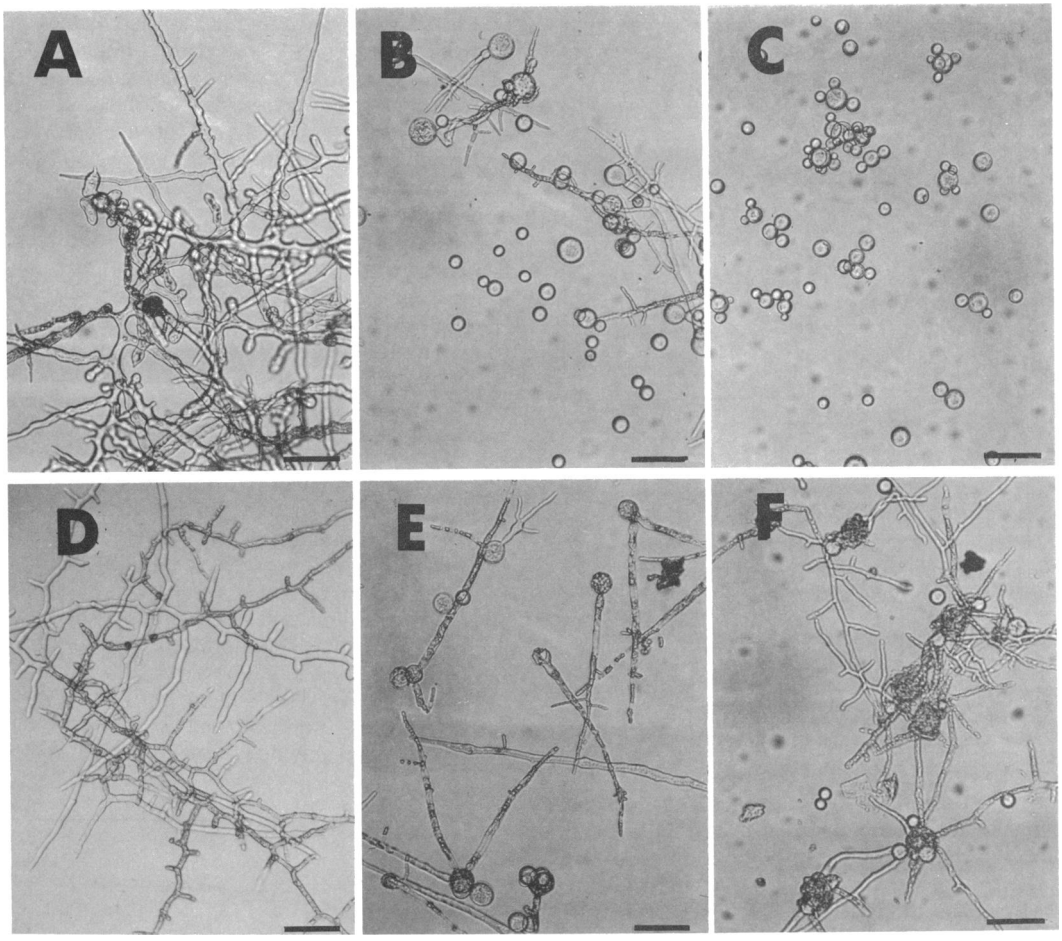

FIG. 1. *Effect of composition of the medium and carbon dioxide tension on the anaerobic morphology of M rouxii NRRL 1894. Letter designations: A, B, and C, 3-days growth on the YPG medium under N_2, 30% CO_2 and 100% CO_2, respectively; D, E, and F, 3-days growth on the basal medium under N_2, 30% CO_2, and 100% CO_2 respectively. Bar indicates 50 μm.*

drolyzed casein supplements to the basal medium were effective only at substrate levels, precluding a catalytic role for these preparations (Fig. 4). Completely yeastlike development was observed only when the concentration of peptone, yeast extract, or enzymatically hydrolyzed casein was equal to or greater than 1, 3, and 3 mg/ml, respectively.

Supplementation with acid-labile amino acids. An obvious difference in composition between acid-hydrolyzed casein (which has no activity) and the substances with activity (peptone, yeast extract, and enzymatically hydrolyzed casein) is the presence, in the latter preparations, of acid-labile amino acids. The following amino acids, known to be acid labile, were added separately or in combination to the basal medium to give a concentration of the L-isomer of 330 μg/ml: trypto-

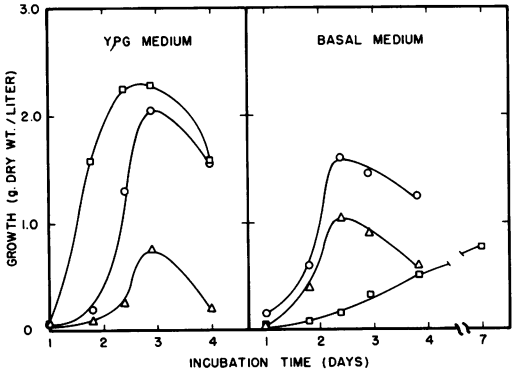

FIG. 2. *Effect of composition of the medium and carbon dioxide tension on anaerobic growth of M. rouxii NRRL 1894. Symbols: □, growth under 100% CO_2; ○, growth under 30% CO_2; △, growth under N_2.*

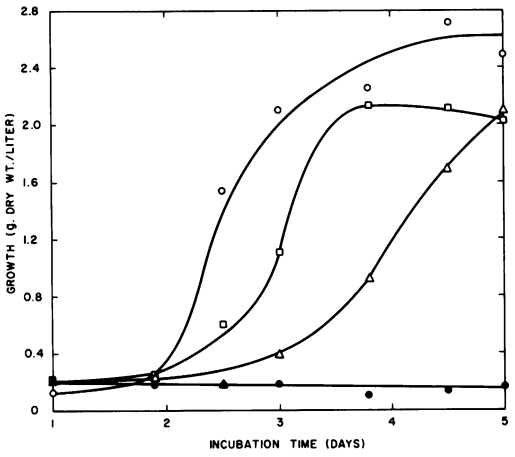

FIG. 3. *Effect of supplements to the basal medium on growth of M. rouxii NRRL 1894 under 100% CO_2. Symbols: ○, growth on peptone-supplemented (10 mg/ml) medium; △, growth on yeast extract-supplemented (3 mg per ml) medium; □, growth on enzymatically hydrolyzed casein-supplemented (6 mg per ml) medium; ●, growth on basal medium.*

phan, methionine, cysteine, serine, phenylalanine, glutamine, and asparagine. Addition of these amino acids to the basal medium did not stimulate growth or promote yeastlike development.

Supplementation with water-soluble vitamins. Other factors common to yeast extract, peptone, and enzymatically hydrolyzed casein are the water-soluble vitamins. (The enzymatically hydrolyzed casein employed was not a vitamin-free preparation.) The following vitamins and vitamin combinations were added to the basal medium (which contained thiamin and nicotinic acid): choline, inositol, biotin, and pyridoxine; choline plus inositol; yeast nitrogen base; and yeast nitrogen base plus ascorbic acid, cyanocobalamin, lipoic acid, choline, and phosphoserine. (In media containing yeast nitrogen base, the initial level of KH_2PO_4 and $MgSO_4$ was held constant by modifying the basal medium to allow for the contribution of these salts by the supplement.) These vitamin-supplemented media did not support yeastlike development under 100% CO_2, nor was the amount of growth at 3 days of incubation significantly increased relative to an unsupplemented control.

Further supplementation of basal medium. *Fusarium oxysporum* has been shown to be capable of anaerobic growth in the presence of nitrate or other compounds that could function as electron acceptors (8). Ergosterol and unsaturated fatty acids have been found to be essential anaerobic growth factors for *Saccharomyces cerevisiae* (1, 2). The unsaturated fatty acid requirement for *S. cerevisiae* could be satisfied by Tween 80. Kihara and Snell (9) devised a nonpeptide-containing supplement (strepogenin supplement) that was superior to tryptic digests of casein as a source of strepogenin activity for *Lactobacillus casei*. With these results in mind, a complete medium was devised which contained, in addition to the acid-labile amino acids and growth factors tested above, the following per liter: xanthine, 20 mg; uracil, 20 mg; guanine, 20 mg; adenine, 20 mg; ergosterol, 10 mg; Tween 80, 10 ml; KNO_3, 3 g; pyridoxal, 10 μg; and pyridoxamine, 10 μg. The following media were then tested for CO_2 growth factor and Y-factor activities: the complete medium, the strepogenin assay medium (9) with and without the strepogenin supplement, and the basal medium with and without the strepogenin supplement. (In this experiment and in subsequent experiments to be discussed the scaled-down incubation system was employed.)

After incubation for 3 days, growth on these media was not significantly greater than that obtained on the unsupplemented basal medium. Growth on media containing the strepogenin supplement was yeastlike, however.

Effect of dialysis. The Y-factor activity in yeast extract, peptone, and enzymatically hydrolyzed casein was completely dialyzable (Table 1). All of

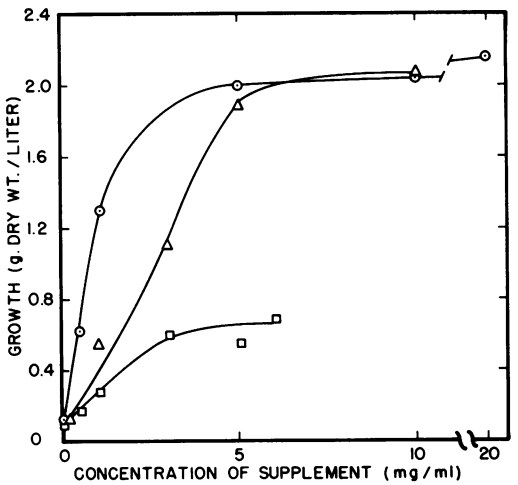

FIG. 4. *Effect of concentration of supplements to the basal medium on growth of M. rouxii NRRL 1894 at 3-days incubation under 100% CO_2. Peptone and yeast extract were added as supplements to the basal medium. Various amounts of enzymatically hydrolyzed casein were substituted for an equivalent amount of acid-hydrolyzed casein component of the basal medium. Symbols: ⊙, growth on peptone-supplemented medium; △, growth on yeast extract-supplemented medium; □, growth on enzymatically hydrolyzed casein-supplemented medium.*

TABLE 1. *Effect of dialysis on the growth-stimulating and Y-factor activities of yeast extract, peptone, and enzymatically hydrolyzed casein*[a]

Addition to the basal medium	Concn (mg/ml)[b]	Growth ratio[c]	Morphology
Peptone	5.0	18	Y
Peptone dialysate	2.9	23	Y
Peptone dialysate (filter sterilized)	2.9	18	Y
Dialyzed peptone	2.1	1.1	MF
Yeast extract	5.0	6	MY
Yeast extract dialysate	3.85	6	MY
Dialyzed yeast extract	1.15	1	MF
EH casein	6.0	6.1	Y
EH casein dialysate	5.05	4.3	Y
Dialyzed EH casein	0.95	1	MF
Pancreatinized casein dialysate	6.0	4.5	MY

[a] Abbreviations: AH casein, acid-hydrolyzed casein; EH casein, enzymatically hydrolyzed casein; Y, yeastlike growth; MF or MY, mostly filamentous or mostly yeastlike growth, respectively; Y + F, approximately equal amounts of filamentous and yeastlike cells.

[b] Data of Fig. 4 show maximal response to 5 mg of peptone or yeast extract per ml and 6 mg of EH casein per ml; concentration of dialysate is the amount that dialyzes from 5 mg of peptone and yeast extract per ml, or from 6 mg of EH-casein per ml.

[c] This ratio was obtained by dividing the dry weight obtained on the supplemented media by that obtained on the basal medium. Cultures were grown for 3 days under 100% CO_2.

the CO_2 growth factor in yeast extract and peptone and most of this activity in enzymatically hydrolyzed casein was also dialyzable. It was found that 77% of the yeast extract, 59% of the peptone, and 84% of the enzymatically hydrolyzed casein was dialyzable.

A pancreatin digest of casein was prepared as described by Sprince and Wooley (15). The casein was extensively dialyzed against distilled water before digestion. As shown in Table 1, the dialysate of the pancreatinized casein, when added to the basal medium, was as active in stimulating growth and yeastlike development as the dialysate of commercially prepared, enzymatically hydrolyzed casein. The activities of peptone dialysate are heat stable in that a filter-sterilized supplement had no more activity than did a supplement autoclaved with the medium.

Effect of gel filtration. The peptone dialysate material was subjected to fractionation on Sephadex G-10. Previous experiments have shown (Fig. 4) that supplementation of 25 mg of peptone to 5 ml of basal medium was sufficient to elicit a maximum CO_2 growth factor response. The 630 mg of peptone dialysate added to the Sephadex column was obtained from 1 g of undialyzed peptone. Since all of the activity of peptone is dialyzable (Table 1), the 630 mg (if added to 5 ml of medium) represents about 40 times the amount necessary for a maximum response. Accordingly, 0.3 ml was taken from each 12-ml Sephadex fraction and added to the medium (final volume, 5 ml) to test for activity. A supplement was also prepared by combining 0.3 ml from each of the fractions. This combined fraction supplement was taken to dryness in vacuo and taken up in 5 ml of medium. Analysis of the fractions for activity (Table 2) revealed that most of the activity is contributed by substances with a molecular weight of less than about 700 since the void material from the column had little activity. Furthermore, no single fraction had CO_2 growth factor activity, although fractions 1, 5, 6, and 7 did partially stimulate yeastlike development. A supplement prepared by combining all of the fractions had almost as much activity as the unfractionated peptone dialysate.

Effect of acid hydrolysis. The effects of acid hydrolysis on the activities of peptone dialysate, yeast extract dialysate, and enzymatically hydrolyzed casein are shown in Table 3. A 24-hr hydrolysis (*see* Materials and Methods) of enzymatically hydrolyzed casein and yeast extract destroyed all of the CO_2 growth factor activity of the former and most of this activity of the latter

TABLE 2. *Effect of gel filtration on the growth-stimulating and Y-factor activities of peptone dialysate*[a]

Addition to the basal medium	Concn (mg/ml)[b]	Growth ratio[c]	Morphology
Peptone dialysate	3.15	13.0	Y
Void volume (V_0)	1.4	1.1	MF
Fraction 1		1.4	Y + F
Fraction 2		1.2	F
Fraction 3		1.3	F
Fraction 4		0.9	F
Fraction 5		1.3	Y + F
Fraction 6		0.9	Y + F
Fraction 7		1.0	Y + F
Fraction 8		1.3	MF
Fraction 9		1.2	MF
Fraction 10		1.3	MF
V_0 plus combined fractions	3.15	10.0	MY
Combined fractions	1.75	8.8	MY

[a] See Table 1 (footnote *a*) for abbreviations.

[b] A 0.3-ml amount from each 12-ml fraction was added to the basal medium (final volume, 5 ml).

[c] See Table 1 (footnote *c*) for method of calculation.

TABLE 3. *Effect of acid hydrolysis on the growth-stimulating and Y-factor activities of peptone dialysate, yeast extract dialysate, and enzymatically hydrolyzed casein*[a]

Addition to the basal medium	Concn (mg/ml)[b]	Growth ratio[c]	Morphology
EH casein	6.0	6.3	MY
EH casein (hydrolyzed for 18 hr at 90 C)	6.0	5.4	MY
EH casein (hydrolyzed for 24 hr)	6.0	0.8	MY
Peptone dialysate	2.9	7.0	Y
Peptone dialysate (hydrolyzed for 24 hr)	2.9	3.7	Y
Peptone dialysate (hydrolyzed for 72 hr)	2.9	1.7	Y
Yeast extract dialysate	3.85	6.0	Y
Yeast extract dialysate (hydrolyzed for 24 hr)	3.85	1.6	Y

[a] See Table 1 (footnote *a*) for abbreviations.
[b] See Table 1 (footnote *b*) for rationale of concentrations employed.
[c] See Table 1 (footnote *c*) for method of calculation.

preparation. Enzymatically hydrolyzed casein which had been subjected to a relatively mild hydrolysis procedure (18 hr at 90 C) had almost as much activity as the unhydrolyzed material. Growth on media supplemented with peptone dialysate material, hydrolyzed for 24 hr, was about one-half that obtained on the medium supplemented with the unhydrolyzed dialysate. A 72-hr hydrolysis, however, destroyed most of the activity of this supplement. Acid hydrolysis did not destroy the Y-factor activity of any of the three preparations.

Amino acid analysis. Analysis of the free amino acid content of peptone, yeast extract, enzymatically hydrolyzed casein, and acid-hydrolyzed casein revealed that these preparations contained 17, 36, 48, and 93% free amino acids, respectively.

The amino acid composition of (i) enzymatically hydrolyzed casein, (ii) a mild and (iii) a strong acid hydrolysate thereof, and (iv) commercially prepared acid hydrolyzed casein is shown in Table 4. Comparison of the free amino acid content of the third and fourth (which had no growth factor activity) with that of the first and second (which had growth factor activity) showed that the latter preparations did not contain any distinctive amino acids. One obvious difference between the second and third was the presence, in the latter, of relatively high levels of proline.

A supplement was prepared which duplicated the free amino acid content of enzymatically hydrolyzed casein. Addition of this supplement (3 mg/ml) to the basal medium did not stimulate growth or support yeastlike development under 100% CO_2. The activity of enzymatically hydrolyzed casein, therefore, cannot be attributed to its free amino acid content.

Effect of specific peptides. Addition (2 mg/ml) to the basal medium of alanylglutamic acid, glycylproline, glycylaspartic acid, glutamylalanine, and prolylglycine, singly or in combination, did not stimulate growth. Glutathione did not have CO_2 growth factor or Y-factor activity. The morphology of growth on media supplemented with glutamylalanine, prolylglycine, or glutamylalanine plus prolylglycine was mixed to the extent that about one-half the growth consisted of budding, yeastlike cells and one half was filamentous. In comparison to the filamentous growth obtained on the basal medium, these dipeptides did partially stimulate yeastlike development.

Y-factor activity. Of all the substances tested, only peptide-containing supplements to the basal medium supported rapid growth under 100% CO_2. The nutritional requirements for yeastlike development under 100% CO_2 are not so specific. All of the peptide mixtures having CO_2 growth factor activity also possessed Y-factor activity. In addition, the basal medium supplemented with the following compounds or mixtures supported

TABLE 4. *Amino acid composition of casein hydrolysates*[a]

Amino acid	EH casein	EH casein (mild, acid hydrolysate)[b]	EH casein (strong, acid hydrolysate)[c]	AH casein
Aspartate	2.13	9.91	9.46	14.24
Threonine	7.42	5.48	5.34	8.28
Serine	5.05	7.58	9.00	11.36
Glutamate	7.65	23.10	26.39	31.72
Proline	3.07	7.05	15.40	23.40
Glycine	0.92	3.85	4.16	6.32
Alanine	5.34	6.30	6.14	9.72
Valine	4.40	8.41	9.75	9.32
Methionine	3.47	3.79	3.07	3.64
Isoleucine	6.06	6.00	7.11	5.40
Leucine	13.30	14.35	12.15	12.36
Tyrosine	2.37	3.45	3.28	3.24
Phenylalanine	5.56	5.70	4.95	3.32
Lysine	10.12	13.37	9.70	10.72
Histidine	1.93	2.25	2.90	3.16
Arginine	4.62	3.89	3.53	3.84

[a] See footnote *a* of Table 1 for abbreviations. Values are expressed as μmoles/25 mg.
[b] Hydrolyzed for 18 hr at 90 C.
[c] Hydrolyzed for 24 hr at 103 C.

yeastlike development, but not rapid growth, under 100% CO_2: acid hydrolysates of dialysates of peptone, yeast extract, and enzymatically hydrolyzed casein; malic acid; succinic acid; fumaric acid; and the strepogenin supplement. No single component of the strepogenin supplement had activity. Furthermore, experiments in which various combinations of the constituents of the strepogenin supplement were tested for activity indicated that only spermine could be omitted from the supplement without loss of at least some of the Y-factor activity.

Since only filamentous growth was obtained on the basal medium containing acid-hydrolyzed casein, it seems unlikely that the activity of peptone dialysate (hydrolyzed for 72 hr) in promoting yeastlike development resided in its amino acid content. Furthermore, it is unlikely that any peptides resisted such prolonged acid hydrolysis [CO_2 growth factor activity was essentially abolished (Table 3)]. Therefore, it appears that some dialyzable component(s) (other than peptides and amino acids) of peptone, yeast extract, and enzymatically hydrolyzed casein possessed Y-factor activity. Investigation of the nature of this material is under way.

DISCUSSION

A number of examples exist where the nutritional requirements of an organism become more complex in the absence of molecular oxygen (1–3, 8, 13). Bartnicki-Garcia and Nickerson (3) have shown that thiamine and nicotinic acid are anaerobic growth factors for *M. rouxii* strain IM80. Strain NRRL 1894 of *M. rouxii* also requires these factors for growth in the absence of molecular oxygen, and the basal medium employed was so supplemented.

The results reported herein are consistent with the view that small, as yet unidentified, peptides are required for rapid growth of *M. rouxii* NRRL 1894 under 100% CO_2. The amount of growth obtained under N_2 or 30% CO_2 is similar in the basal medium and in a medium containing peptides (Fig. 2), whereas under 100% CO_2 growth is stimulated 4- to 23-fold by the addition of peptide-containing mixtures to the basal medium. These peptide-containing mixtures must be present in substrate levels for maximal activity. Furthermore, the CO_2 growth factor activity of these preparations is dialyzable and mostly destroyed by acid hydrolysis.

Amino acid analysis of a vigorous acid hydrolysate and a relatively mild acid hydrolysate of enzymatically hydrolyzed casein showed that the former had significantly higher levels of proline. Since only the latter preparation exhibited CO_2 growth factor activity, the peptides involved may be particularly rich in proline.

The growth-factor activity of some complex media has been attributed to contaminating fatty acids (6). A rigorous extraction of lipids from peptone failed to remove its growth factor activity for *M. rouxii*. Ashing this preparation did, however, destroy activity.

Fractionation of peptone by gel filtration revealed that most of the activity could be attributed to peptides with a molecular weight of less than about 700. Although the experimental technique employed was sufficiently sensitive to detect growth-factor activity if the activity were contributed by only one or a few peptides, no single fraction contained detectable activity. However, the combination of all the fractions exhibited most of the original activity. These results suggest that either there are several peptides, all of which are required for rapid growth, or that the activity observed is due to the cumulative effects of a series of low-molecular-weight peptides.

A large number of peptides have been isolated or synthesized that have partial strepogenin activity for growth of *L. casei* (*compare* 14). The diverse structures of the active peptides suggest that strepogenin activity cannot be attributed to a particular chemically defined peptide but is a property of a wide variety of peptides (19). In view of this lack of specificity, it is not surprising that we were unable to isolate a specific peptide having all of the CO_2 growth factor activity.

From the work of Kihara and Snell (9, 10), it appears that the strepogenin activity of certain peptides is due to their contribution of amino acids in a readily assimilable form. Further, it has been shown that independent uptake mechanisms exist for glycine and for glycyl peptides in *L. casei* (11). Since the basal medium employed in our experiments contained the amino acids present in acid-hydrolyzed casein, high CO_2 tensions apparently impair the utilization of some essential amino acid(s) but not of their peptides. Thus, growth was rapid in the basal medium under 30% CO_2 but addition of peptide-containing mixtures was required for rapid growth under 100% CO_2.

Kihara and Snell (9) described a nonpeptide-containing supplement that is superior to protein digests for supporting rapid growth of *L. casei*. Addition of this supplement to the basal medium or to a strepogenin assay medium (9) did not stimulate growth of *M. rouxii*. This, however, is not surprising in view of the vastly different environmental conditions under which *M. rouxii* was grown.

The nature of the Y-factor activity of the various supplements tested is more difficult to define. Whereas only peptide mixtures were

active in stimulating growth under 100% CO_2, a wide array of compounds and mixtures showed Y-factor activity (e.g., malic acid, peptone dialysate, acid-hydrolyzed peptone dialysate, strepogenin supplement). A subtle nutritional balance may be requisite for yeastlike development of *M. rouxii* NRRL 1894 under CO_2.

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