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Effect of Oxygen on Morphogenesis and Polypeptide Expression by *Mucor racemosus*

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The morphology of *Mucor racemosus* in cultures continuously sparged with nitrogen gas was investigated. When appropriate precautions were taken to prevent oxygen from entering the cultures, the morphology of the cells was uniformly yeastlike irrespective of the N_2 flow rate. When small amounts of oxygen entered the cultures the resulting microaerobic conditions evoked mycelial development. Polypeptides synthesized by aerobic mycelia, microaerobic mycelia, anaerobic yeasts, and yeasts grown in a CO_2 atmosphere were compared by two-dimensional gel electrophoresis. The results indicated that a large number of differences in polypeptide expression exist when microaerobic mycelia or anaerobic yeasts are compared with aerobic mycelia and that these alterations correlate with a change from an oxidative to a fermentative metabolic mode. Relatively few differences in polypeptide composition exist when microaerobic cells are compared with anaerobic cells, but these changes correlate with a change from the mycelial to the yeast morphology. We hypothesize that oxygen regulates the expression of polypeptides involved in both the metabolic mode and in morphogenesis.

Dimorphic species of the fungus *Mucor* can grow in two alternative vegetative growth modes. The cells exist either as unicellular, spherical, budding yeasts or as filamentous hyphae. Relative to dimorphism, carbon and energy metabolism in *Mucor* have received more experimental attention than virtually any other area. The tendency of a given species of *Mucor* to undergo either hyphal or yeast morphogenesis depends upon an interplay of environmental factors which include the presence of carbon dioxide, oxygen, and a fermentable energy source. In general, yeast development occurs under conditions which favor a fermentative mode of metabolism (anaerobiosis and high hexose concentrations), while mycelial development occurs under conditions which favor an oxidative mode of metabolism (aerobic conditions in the presence of oxidizable energy sources) (14).

A notable exception to the apparent correlation between energy metabolism and morphogenesis was reported by Mooney and Sypherd (8). They showed that the morphology of *Mucor racemosus* cultures continuously sparged with nitrogen was dependent upon the flow rate of N_2 through the culture. At relatively low flow rates (<0.5 ml of N_2 per min per ml of culture) growth was mycelial, whereas at high flow rates (>2 ml of N_2 per min per ml of culture) growth was yeastlike. Growth under both conditions was believed to be rigorously anaerobic. Their results were interpreted as evidence of a volatile factor (produced by cells) which either stimulates mycelial development or blocks yeast development. Paznokas and Sypherd (10) subsequently demonstrated that the yeasts and mycelia generated under an N_2 atmosphere possessed equal respiratory capacity. Quite correctly, then, it was argued that the development of respiratory capacity was not an obligatory correlate of morphogenesis.

The availability of a means to generate either yeast or mycelial cells under virtually identical, presumably anaerobic conditions provoked us to study differential gene (polypeptide) expression which might accompany morphogenesis under conditions of high and low flow rates of N_2. This had not been reported in the previous study of Hiatt et al. (4). During the course of our study, several findings emerged. We demonstrated that small amounts of oxygen enter the growth medium under the conditions of low-flow-rate nitrogen previously reported and that the resulting microaerobic conditions are apparently responsible for the mycelial growth. In addition, a comparison of two-dimensional gels of polypeptides from aerobic mycelial, microaerobic (low-flow-rate N_2) mycelia, and anaerobic (high-flow-rate N_2) yeasts showed (i) that the pattern of polypeptide expression from aerobic mycelia differs substantially from that of microaerobic mycelia and that these alterations correlate with a physiological change from an oxidative metabolic mode to a fermentative mode; and (ii) that anaerobic yeasts show relatively few changes in polypeptide expression when compared with microaerobic mycelia; both of these forms are essentially fermentative. Thus, it is apparent that the change in metabolic mode places a greater demand for changes in the expression of major cell polypeptides than does the yeast-to-mycelia transition. Nevertheless, the presence of a small number of polypeptides whose expression is altered in microaerobic mycelia versus anaerobic yeasts suggests that alterations in polypeptide expression are involved in morphogenesis.

MATERIALS AND METHODS

Organism and medium. *M. racemosus* ATCC 1216B was used throughout. All experiments were performed in yeast extract (0.3%)–peptone (1.0%)–glucose (2.0%) medium (YPG) adjusted to pH 4.5 with H_2SO_4. Growth was at 28°C. Cultures were inoculated with sporangiospores at a concentration of 2×10^5 spores per ml for CO_2- and N_2-grown cultures and 1×10^6 spores per ml for aerobic cultures. Proteins were labeled by the addition of 5 μCi of [^{14}C]leucine per ml.

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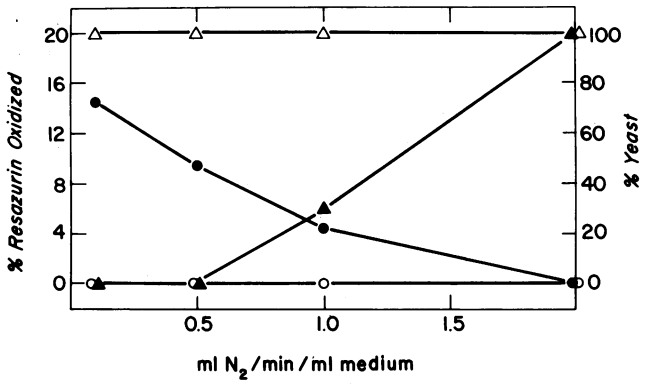

FIG. 1. Effect of nitrogen flow rate on the redox potential (circles) and the morphology (triangles) of *M. racemosus*. Open figures represent the use of Saran tubing in the sparging system. Closed figures represent the use of Teflon tubing in the system.

Culture conditions. All experiments were performed in 125-ml nephelometer flasks containing 20 ml of YPG medium. The flasks were agitated at 50 rpm on a rotary shaker. Linde high-purity-grade N_2 was sequentially passed through hot copper turnings in a glass tube in a Sargent-Welch S-36517 furnace, a gas-washing bottle (Corning no. 31770) containing distilled water, and into a metering manifold consisting of Nupro SS-2SX metering valves connected in series. Individual metering valves were connected by 3-ft (91-cm) lengths of tubing (Telfon or Saran) (1/8-in. [0.32-cm] outer diameter by 0.031-in. [0.08-cm] inner diameter) to Pasteur pipettes which passed through a butyl rubber stopper in the nephelometer flask. The rubber stopper was provided with a glass-tubing gas outlet. Back flow of gas was prevented by connecting the gas outlet to a length of flexible rubber tubing which extended beneath the surface of distilled water contained in a second flask. The gas tank pressure regulator, the tube containing the copper turnings, the gas-washing bottle, and the manifold were interconnected by 3-ft (91-cm) lengths of Teflon or Saran tubing (118-in. outer diameter by 0.031-in. inner diameter). Glass-to-tubing connections were made via Glenco Omnifit tubing connectors. A pressure of 5 lb/in^2 was maintained in the gassing manifold.

In the redox potential experiments, the medium contained 0.001% resazurin and 50 μg of cysteine per ml. The medium was prebubbled with 10 ml of N_2 per min per ml of medium for 2 h and was then brought to a gentle boil over a Bunsen burner to reduce the resazurin. The flow rate was then reduced to the indicated value, and the flask was placed in a water bath. The absorbance of the medium at 550 nm was periodically monitored in a Spectronic 20 colorimeter until no further change was observed (3 h). The percentage of resazurin reduced was determined from a standard curve of oxidized resazurin.

For experiments involving the effect of N_2 flow rate on morphology, 20 ml of YPG was prebubbled with N_2 for 2 h and then gently boiled over a Bunsen flame. The medium was cooled and inoculated with sporangiospores, and the flow rate was adjusted to the indicated value. Growth proceeded for 18 h at 28°C, and microscopic morphology was scored at that time.

In experiments involving a CO_2 atmosphere or aerobic conditions, cultures were sparged with Linde carbon dioxide or compressed air at flow rates of 5 volumes of gas per min per volume of culture of 10 volumes of air per min per volume of culture, respectively. The same gassing system was used for these experiments as described for the N_2 experiments except that the copper turnings and furnace were omitted.

For experiments involving the examination of polypeptides, cultures labeled continuously from spores were grown to an optical density of 0.4 at 490 nm (mid-log phase). In N_2-flow-rate shift experiments, the shift was initiated at a point in growth such that the final optical density after a 3-h period was 0.4.

Electrophoresis of proteins. Cells were harvested by centrifugation and washed twice with a buffer containing 0.01 M Tris hydrochloride (pH 7.4), 5 mM $MgCl_2$, and 2 mM phenylmethylsulfonyl fluoride. Cells were broken by vortexing in the presence of 1 g of acid-washed sand (Ottowa sand, Fisher Scientific Co.) per ml of cell suspension for 5 min. Greater than 90% breakage of cells occurred as determined by microscopic analysis. RNase and DNase were added to the broken-cell extract to give a final concentration of 4 μg/ml, and the extract was incubated at 0°C for 30 min. The proteins were solubilized by urea as described by O'Farrell (9). Urea was added to the cell extract to bring the concentration to 9.5 M. An equal volume of buffer containing 9.5 M urea, 2% (wt/vol) Nonidet P-40, 2% ampholtyes (1.6%, pH 5 to 7; 0.4%, pH 3.5 to 10), and 5% β-mercaptoethanol was added and incubated at room temperature until the urea was solubilized. Insoluble material was removed by centrifugation in an Eppendorf microcentrifuge, and samples were either run immediately in the first-dimension gel or frozen at −70°C until use.

Two-dimensional polyacrylamide gel electrophoresis was performed as described by O'Farrell (9) with modifications as described by Pedersen et al. (11). A total of 10^5 cpm were applied to the first dimension of gels to be autoradiographed, and 100 μg of protein was applied in the case of stained gels. Protein was determined by the Lowry method (7). The first dimension was run in tubes 1.5 mm in inner diameter and 14 cm long and had a pH range of 5.0 to 7.0. Prerunning the unloaded gels was not done. The gels were electrophoresed for 5,600 Vh. The second dimension was a 10% acrylamide-sodium dodecyl sulfate (SDS) gel, and electrophoresis was conducted at a constant power of 2.5 W per gel. Gels were stained with Coomassie brilliant blue R-250 (11) and dried. Dried gels were exposed to Kodak XR-5 X-ray film at −70°C for 7 weeks.

Growth yield and ethanol-glucose measurements. Molar growth yields (Y_{glu}) were determined on cells growing under the stated conditions in YPG medium with several concentrations of glucose. The relationship between glucose concentration and dry weight at the stationary phase was linear, and the slope of this line was used to determine Y_{glu}.

Ethanol was measured in culture supernatants according to Sigma Technical Bulletin 331 UV. Glucose was determined according to Sigma Technical Bulletin 510.

Chemicals. [^{14}C]leucine was purchased from Amersham-Searle. SDS, manufactured by BDH, was purchased from Gallard Schlesinger. Ampholytes were purchased from LKB. Urea, ultrapure grade, was purchased from Schwarz/Mann. Molecular weight standards were purchased from Bio-Rad. Acrylamide, bisacrylamide, TEMED (*N,N,N',N'*-tetramethylethylenediamine), and Coomassie brilliant blue-R 250 were all purchased from Eastman Organic Chemicals. Phenylmethylsulfonyl fluoride, RNase, DNase, and agarose were purchased from Sigma Chemical Co.

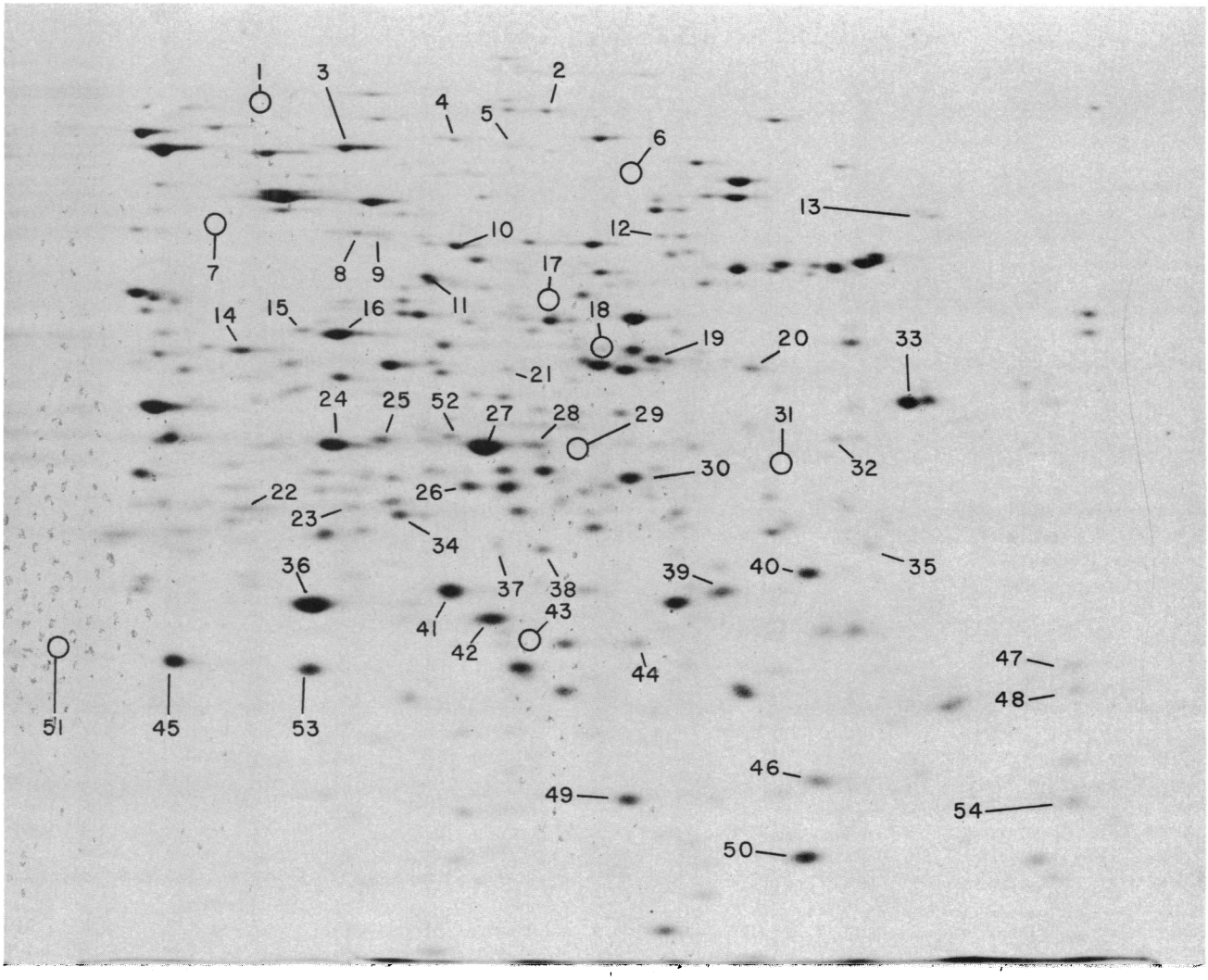

FIG. 2. Autoradiogram of two-dimensional gel of polypeptides from aerobically grown mycelial cells. The first dimension was a pH 5 (right) to pH 7 electrofocusing gel. The first dimension was loaded with 10^5 cpm of incorporated [^{14}C]leucine. The numbered polypeptides are discussed in the text.

RESULTS

Nitrogen flow rate, dissolved oxygen, and morphogenesis. In experiments in which we attempted to duplicate the results of Mooney and Sypherd (8) it was noticed that if Teflon tubing was used to convey gas to the culture flasks, the morphology varied as a function of flow rate in the manner they reported. If, however, the Teflon tubing was replaced with Saran tubing, the morphology was independent of the N_2 flow rate. We suspected that these findings might be indicative of small amounts of oxygen in the cultures at low flow rates when Teflon tubing was used.

Figure 1 shows the results of an experiment designed to determine the redox potentials of the medium at low N_2 flow rates and at high N_2 flow rates when Saran and Teflon tubings were used. With Teflon tubing, the results show that at low N_2 flow rates (0.1 ml of N_2 per min per ml of medium) the resazurin redox indicator was 15% oxidized, corresponding to an E_h of -0.07 V. Under these conditions the cells were uniformly mycelial after 18 h of growth. As the flow rate was increased, the percentage of resazurin oxidized dropped to undetectable levels ($E_h < 0.10$ V) and the population was uniformly yeastlike. When Saran tubing was used, no oxidation of resazurin was detectable irrespective of the N_2 flow rate. In addition, the cells invariably grew in the yeast morphology. Thus, when Saran tubing was used,

TABLE 1. Growth yield (Y_{glu}) and ethanol produced per glucose consumed as a function of growth conditions and morphology

Growth conditions[a]	Morphology	Ethanol produced/ glucose consumed	Molar growth yield[b]
Aerobic	Mycelium	0.10	81.6
Microaerobic	Mycelium	1.86	22.4
Anaerobic	Yeasts	1.91	21.7

[a] Microaerobic and anaerobic conditions were imposed by varying the flow rate of nitrogen gas through the culture in the manner described in the text, using Teflon tubing in the gassing system.

[b] Expressed in grams (dry weight) per mole of glucose from cells grown in YPG medium.

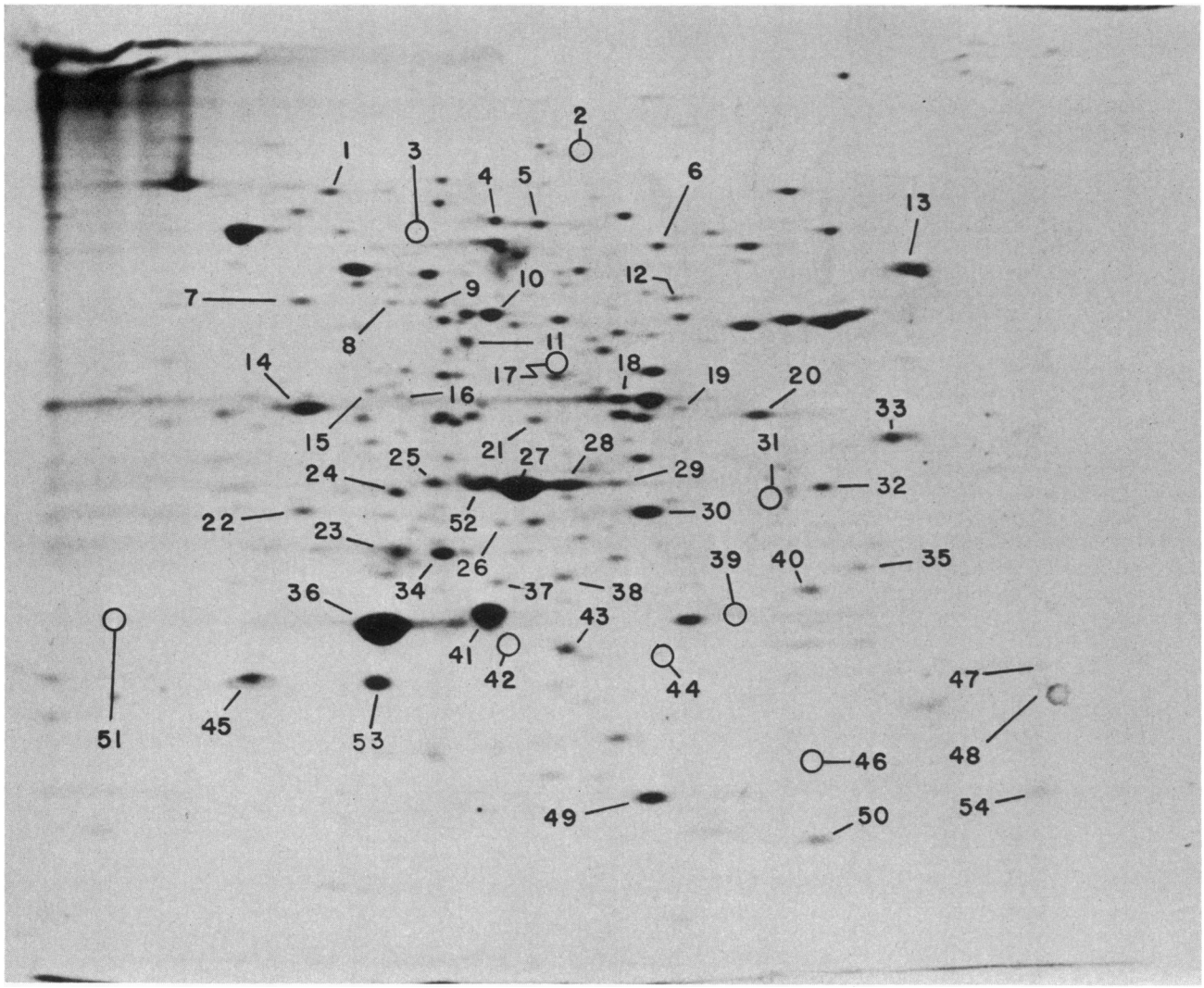

FIG. 3. Autoradiogram of two-dimensional gel of polypeptides from yeasts grown in a CO_2 atmosphere. The first dimension was a pH 5 (right) to pH 7 electrofocusing gel. The first dimension was loaded with 10^5 cpm of incorporated [^{14}C]leucine. The numbered polypeptides are discussed in the text.

the morphology was independent of N_2 flow but dependent on redox potential or a related parameter such as dissolved oxygen. Measurements of dissolved oxygen with a Clark-type electrode (Yellow Springs Instruments), while at the limit of sensitivity of the instrument, revealed that when Teflon tubing was used, there was 1.2 μM dissolved oxygen at 0.1 ml of N_2 per min per ml of medium but ≤ 0.2 μM at 2.0 ml/min per ml of medium. When Saran tubing was used, there was ≤0.2 μM dissolved oxygen over the range of flow rates used.

These results support the view that the mycelial growth in *Mucor* cultures grown under constant N_2 sparging at low flow rates is due to small amounts of oxygen present in the cultures. Presumably, at low flow rates when Teflon is used, sufficient oxygen is able to diffuse through the tubing walls to cause hyphal development. The presence of small amounts of oxygen can be detected by the use of redox dyes such as resazurin. The apparent accumulation of oxygen (microaerobic conditions) is prevented (when using Teflon) at elevated N_2 flow rates, and this is manifested in the yeast morphology. Oxygen is known to permeate Saran tubing poorly (13) and when used in sparging *Mucor* cultures at either high or low flow rates of N_2, oxidation of resazurin could not be detected and yeast development occurred. When resazurin was incorporated in the medium at 0.0001% as utilized by Mooney and Sypherd (8), at low flow rates of N_2 with Teflon tubing, resazurin oxidation could not be detected by visual inspection. Only at 0.001% dye did the oxidation become apparent visually under these conditions. In the remainder of the paper, for the sake of convenience, yeast cells generated by using the sparging system with Teflon tubing and a flow rate of 2.0 volumes of N_2 per volume of culture per min are referred to as anaerobic yeasts. Mycelia generated with the same sparging system at a flow rate of 0.1 volumes of N_2 per volume of culture per min are referred to as microaerobic mycelia.

Sample extraction and preparation. Cell samples were broken by vortexing with acid-washed sand and extracting the broken cells with urea and Nonidet P-40 essentially by the method of O'Farrell (9). This procedure solubilized 89% of the trichloroacetic acid-precipitable counts. In experiments in which heat and SDS were used to extract proteins

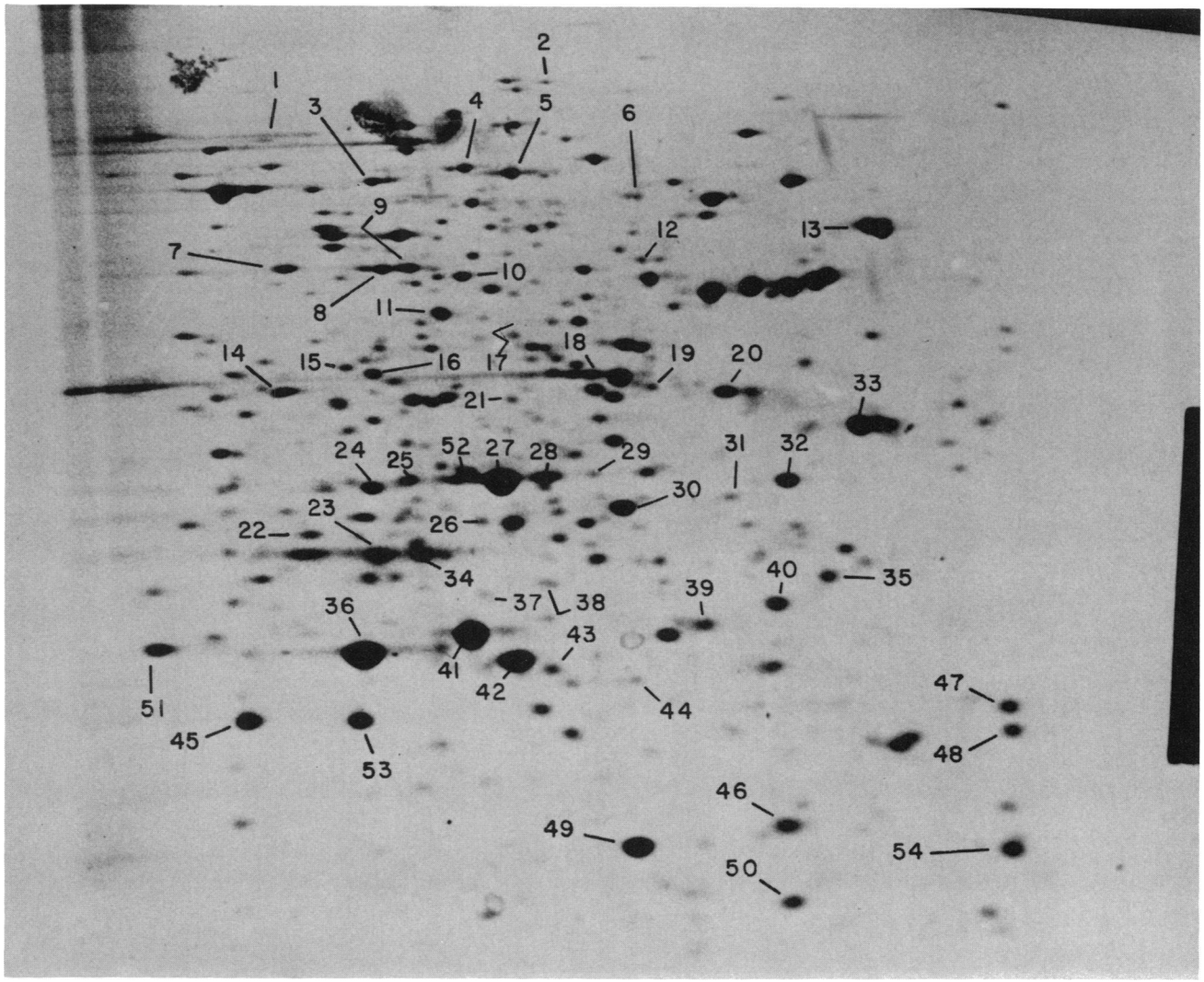

FIG. 4. Autoradiogram of two-dimensional gel of polypeptides from mycelia grown microaerobically by sparging N_2 gas through the culture (see text). The first dimension was a pH 5 (right) to pH 7 electrofocusing gel. The first dimension was loaded with 10^5 cpm of incorporated [^{14}]leucine. The numbered polypeptides are discussed in the text.

by the method of Ames and Nikaido (1) in the ratio of SDS/protein of 1.3:1, 95% of the counts were solubilized. Thus, no substantial benefit was gained by utilizing SDS. In addition, a considerable alteration in the migration of polypeptides in the first dimension of the gels was noticed, resulting in decreased differences in the isoelectric points of most polypeptide species (data not shown). Interestingly, polypeptides 42 and 43 (see below) could not be resolved when SDS was used in the extraction procedure. For these reasons, the Nonidet P-40–urea extraction procedure was used in all the gels described in the text.

Polypeptides from aerobic mycelia and CO_2-grown yeasts. Figure 2 is an autoradiogram of *Mucor* proteins from mycelial cells grown from spores under aerobic conditions in the presence of [^{14}C]leucine. These cells were 100% mycelial at the point of cell harvest. Figure 3 is an autoradiogram of proteins from yeast cells grown from spores in the presence of a carbon dioxide atmosphere. Comparison of Fig. 2 and 3 shows that polypeptides 2, 3, 39, 42, 44, and 46 are present only in the aerobic mycelial cells. Polypeptides 16, 19, 24, 26, 33, 40, 47, 48, 50, and 54 are present in both conditions but are found in greater amounts in the aerobic mycelial cells. Conversely, polypeptides 1, 6, 7, 18, 29, and 43 are unique to the CO_2-grown yeast cells, and polypeptides 4, 5, 10, 13, 14, 20, 22, 23, 25, 27, 28, 32, 34, 36, 41, 49, and 52 are found in greater amounts in the yeast cells. As shall be shown later, many polypeptides found in greater amounts in the CO_2-grown yeasts compared with aerobic cells were found in greater amounts in all cells grown in oxygen-depleted conditions irrespective of morphology.

Cells grown aerobically versus those grown in a CO_2 atmosphere not only exhibited different morphology, they differed in growth rates. CO_2-grown yeast cells had a doubling time of 4 h, while aerobic cells doubled every 1.5 h (data not shown). In addition, it has been shown that these cells differ in carbon catabolism, in terms of flux and distribution through fermentative and oxidative metabolic pathways (5). Therefore, comparison of these two figures cannot differentiate polypeptides unique to one morphology from those that reflect metabolic- and growth-dependent differences between the cells.

Polypeptides of yeastlike cells generated under a high flow of

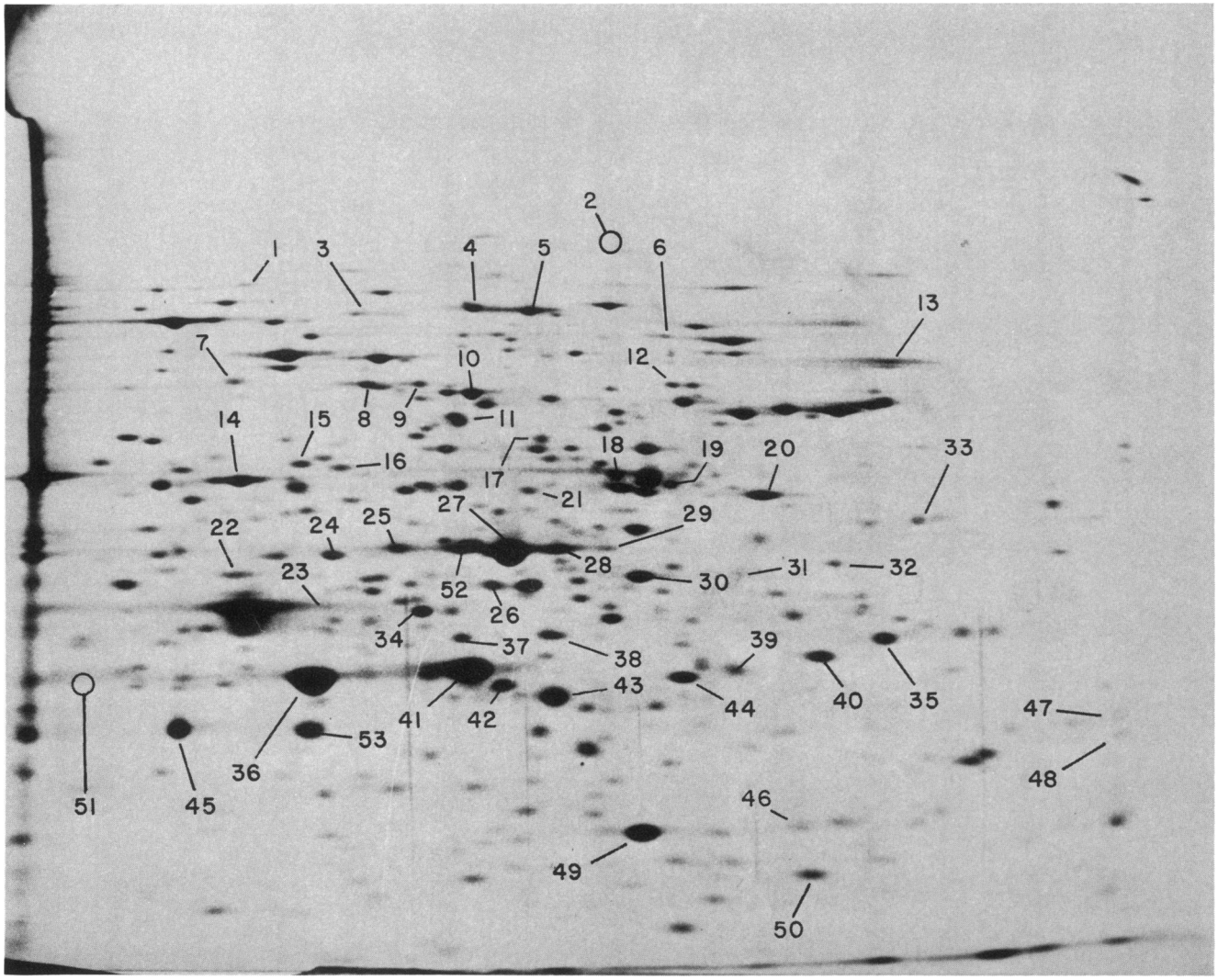

FIG. 5. Autoradiogram of two-dimensional gel of polypeptides from yeasts grown anaerobically by sparging N_2 gas through the culture (see text). The first dimension was a Ph 5 (right) to pH 7 electrofocusing gel. The first dimension was loaded with 10^5 cpm of incorporated [^{14}C]leucine. The numbered polypeptides are discussed in the text.

N_2 and of mycelial cells generated under a low flow of N_2. Figures 4 and 5 show the pattern of polypeptides synthesized by microaerobic mycelia and anaerobic yeasts. In each case, cells were continuously labeled from spores. These cells had identical growth rates (1.8-h mass-doubling time), exhibited comparable respiratory capacity (10), and were equally fermentative in culture as measured by an ethanol produced/glucose consumed ratio of 1.9 and molar growth yield (Table 1). Additionally, the rate of incorporation of [^{14}C]leucine into trichloroacetic acid-precipitable material and the specific activity of the synthesized protein were identical (data not shown). Thus, notwithstanding the apparent small difference in cultural oxygen concentration, the conditions used to generate these cells represent the most similar set of environmental conditions capable of inducing the yeast and mycelial morphologies in *M. racemosus* and are thus most likely to reveal polypeptides which are associated with morphogenesis.

Inspection of Fig. 4 or Fig. 5 indicates that both bear more similarity to the autoradiogram of CO_2-grown cells (Fig. 2) than they do to the autoradiogram of aerobically grown cells (Fig. 1). In either anaerobic yeasts or microaerobic mycelia and in CO_2-grown yeasts polypeptides 4, 5, 6, 10, 13, 14, 18, 20, 22, 23, 25, 27, 28, 29, 32, 34, 36, 41, 49, and 52 were exclusively present or present in greater amounts in the oxygen-depleted cells (CO_2 sparged, microaerobic or anaerobic) when compared with the aerobically grown cells. Thus, considerable qualitative and quantitative differences exist between O_2-deprived cells and aerobic cells irrespective of the morphology of the cells. The most striking difference between aerobic and oxygen-deprived cells is the increase in the amount of a few polypeptide species such as 27, 41, and 36 to a relatively large fraction of the total cellular protein.

Comparison of microaerobic mycelial polypeptides (Fig. 4) with those from anaerobic yeasts (Fig. 5) shows that polypeptides 2, 23, 32, 33, 42, 46, 47, 48, and 51 are present either exclusively or in higher quantities in the mycelial cells, while polypeptides 17, 37, 38, and 43 are present in higher relative quantities in the yeast cells.

If the polypeptides which differ in anaerobic yeasts and microaerobic mycelia grown from spores are obligately

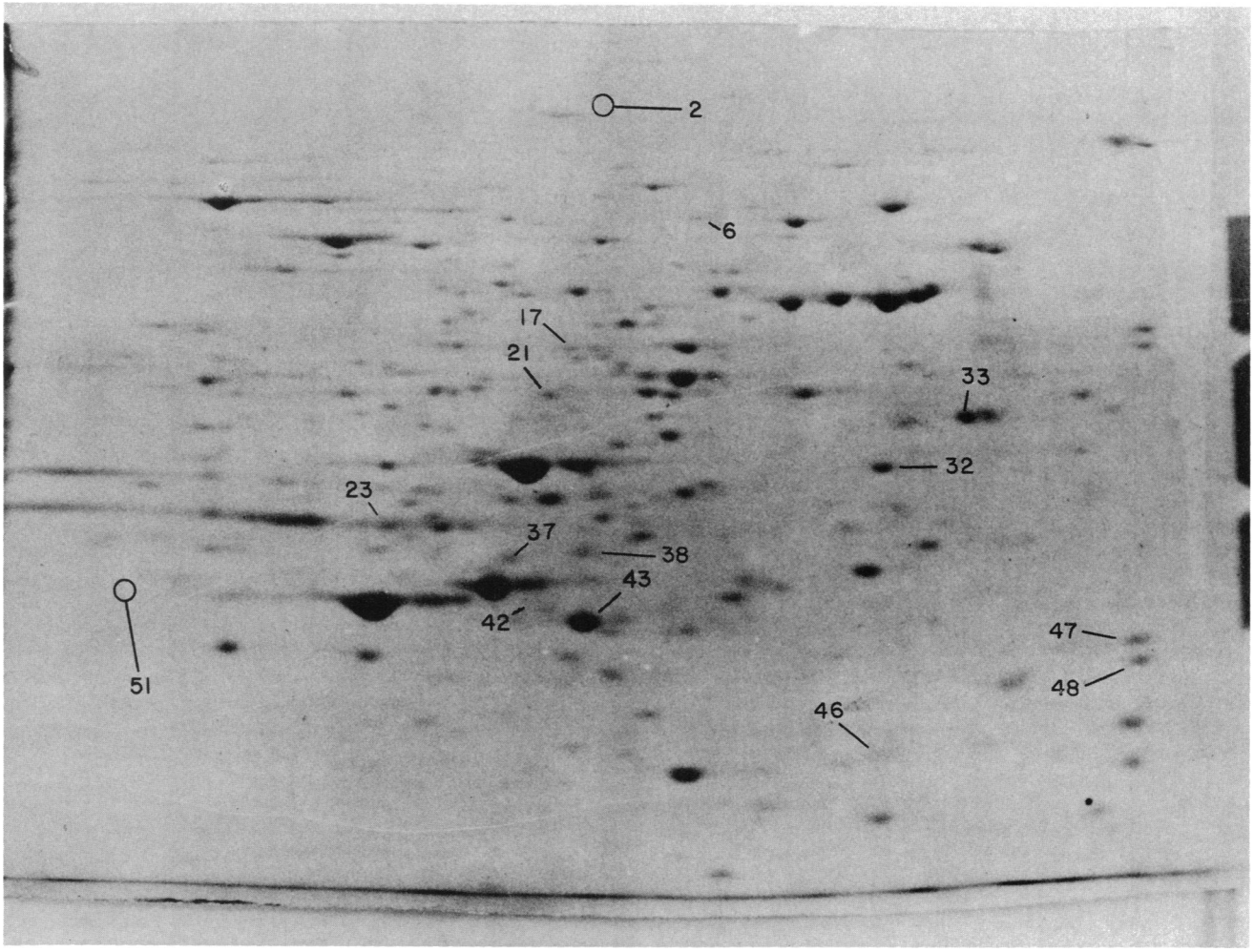

FIG. 6. Stained two-dimensional gel of polypeptides from yeasts grown anaerobically in an N_2 atmosphere. The first dimension was loaded with 100 μg of protein. The numbered proteins are discussed in the text.

associated with the morphological change, the same alterations in polypeptide complement should also occur when dimorphic transitions are evoked by shifting nitrogen flow in vegetative cultures. Figure 6 shows the stained gel of unlabeled anaerobically grown yeasts before shifting the culture to a low flow rate of N_2 (microaerobic conditions). At the point of shift, [^{14}C]leucine was added and growth was continued for 4 h. At the time of harvest, the cells had converted to the mycelial morphology. Figure 7 is the autoradiogram from the postshift cells. For the polypeptides which were present in greater amounts in microaerobic mycelia grown from spores, analysis of the gel pattern from the postshift mycelia indicates that polypeptides 23, 42, 47, 48, and 51 are found in increased amounts when compared with the polypeptides in anerobic yeasts generated from spores. Polypeptides 2, 32, 33, and 46, however, were not appreciably altered in amount in the postshift mycelia when compared with anaerobic yeasts generated from spores. Thus, it is unlikely that changes in the relative amounts of this latter group of polypeptides are obligately associated with morphogenesis. Likewise, comparison of the preshift stained gel with the postshift gel indicates that polypeptides 47 and 48 are present in about equal amounts before and after the shift. Of the polypeptides which were present in decreased amounts in microaerobic mycelia grown from spores, polypeptide 43 was also found in relatively low amounts in the postshift mycelia although it was a prominent species in the preshift stained gel and in anaerobic yeasts generated from spores. Polypeptides 17, 37, and 38 were apparently made in similar relative amounts in postshift mycelia when compared with anaerobic yeasts generated from spores (Fig. 5). Summarizing the data derived from cells grown anaerobically and microaerobically from spores and from the shift experiment leads to the conclusion that changes in the relative amounts of polypeptides 23, 42, 43, and 51 correlate well with morphogenesis. It is also noteworthy to point out that the relative changes in polypeptides 23, 42, and 43 illustrated by anaerobic yeasts and microaerobic mycelia in Fig. 4 through 7 are apparent in CO_2-grown yeasts and aerobic mycelia. Thus, polypeptides 23 and 42 were characteristically found in higher relative amounts in all mycelial cultures and polypeptide 43 was elevated in all of the yeast cultures.

Relationship between polypeptides 42 and 43. Polypeptides 42 and 43 have apparent molecular weights of about 35×10^3 and are close enough in isoelectric point to possibly be derivatives of one another by posttranslational modification(s). To determine whether they represent distinct or

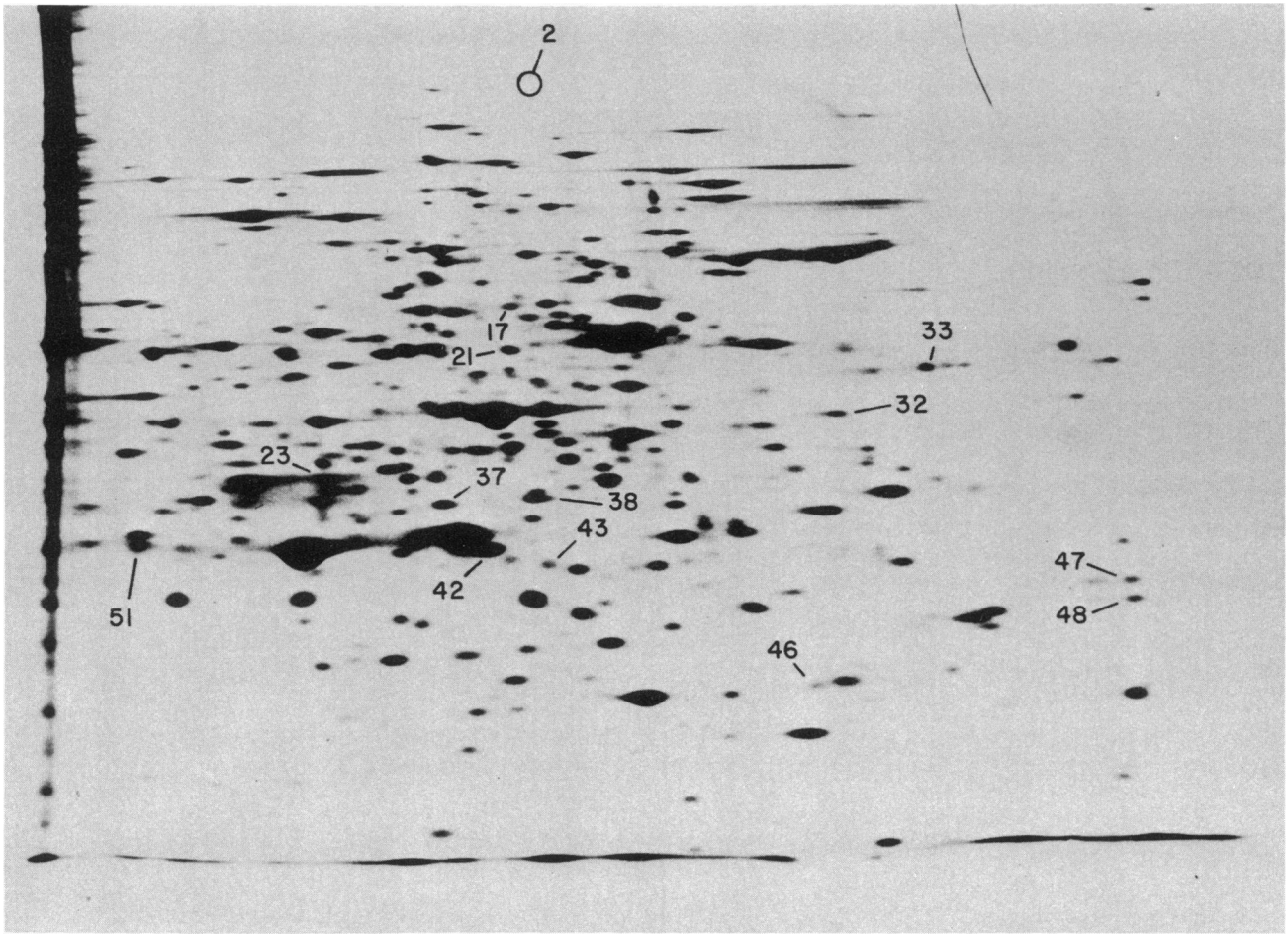

FIG. 7. Autoradiogram of two-dimensional gel of polypeptides from cells (mycelia) 4 h after a shift from anaerobic conditions in an N_2 atmosphere to microaerobic conditions. Concomitant with the shift, the culture was labeled with [^{14}C]leucine. The first dimension was loaded with 10^5 cpm. The numbered proteins are discussed in the text.

related polypeptides, a series of pulse-chase experiments were performed. Figure 5 shows the gel from [^{14}C]leucine-labeled anaerobic yeasts, while Fig. 8 shows the gel from the same culture 4 h after the labeled leucine was removed and it had been shifted to a low flow rate of N_2 (microaerobic conditions). Comparison of the two gels indicates that the relative amounts of polypeptides 42 and 43 stayed constant, indicating that 42 is not derived from 43.

Figure 4 is the autoradiogram from [^{14}C]leucine-labeled microaerobic mycelia, while Fig. 9 shows the gel from a portion of the same culture 4 h after the labeled leucine was removed and the culture was shifted to a high flow rate of N_2 (anaerobic conditions). Comparison of Fig. 4 and 9 indicates that the relative proportions of polypeptides 42 and 43 stayed constant, indicating that polypeptide 43 is not derived by posttranslational modification of polypeptide 42.

Molar growth yield and ratio of ethanol produced/glucose consumed. Table 1 shows the molar growth yields (Y_{glu}) and ratio of ethanol produced/glucose consumed for cells grown aerobically and microaerobically as mycelia and anaerobically as yeasts. Aerobic mycelial cells produced little ethanol and had growth yields about four times greater in batch culture than either microaerobic mycelia or anaerobic yeasts. The ethanol/glucose ratios and the Y_{glu} of both microaerobic mycelia and anaerobic yeasts were typical of cells growing with a classical ethanol fermentation. Molar growth yields of 21 are typical of fermentative pathways which yield 2 ATPs per mol of glucose fermented (3).

DISCUSSION

The finding that mycelial morphogenesis requires at least small amounts of oxygen was surprising and we believe significant. Since the publication of the original work on the influence of nitrogen flow rate on morphogenesis (8), several laboratories have had difficulty in reproducing the work (P. S. Sypherd, personal communication; S. Bartnicki-Garcia and J. Ruiz-Herrera, personal communication). In light of our findings, it seems likely that these discrepancies are due to variability in the amounts of oxygen which can permeate the sparging systems used in different laboratories.

At present, we can only speculate as to the basis for the apparent oxygen requirement for mycelial development. It is clear, however, that oxygen need not be present in large amounts to satisfy the requirement. Since microaerobic mycelia and anaerobic yeasts have essentially the same molar growth yield on glucose, it is unlikely that oxidative energy metabolism is making a significant contribution to ATP production. In addition, as measured by ethanol/glucose ratio measurements, both forms are equally fermentative. Thus, it is unlikely that the mycelial morphogenesis is directly related to a major alteration in the mode of energy metabolism. This conclusion is consistent with work in other

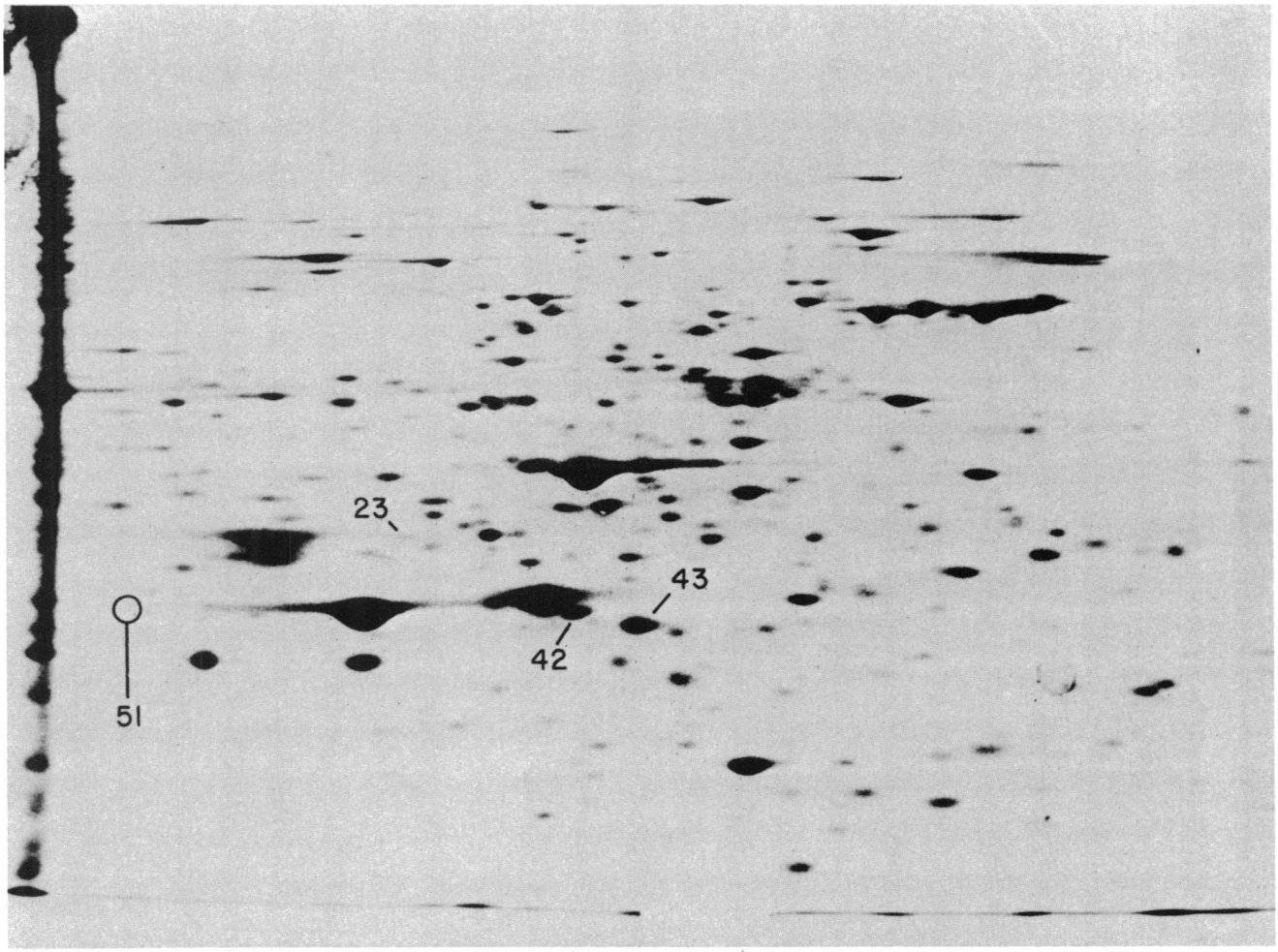

FIG. 8. Autoradiogram of two-dimensional gel of polypeptides from cells 4 h after a shift from anaerobic conditions in an N_2 atmosphere to microaerobic conditions. The preshift culture was labeled with [^{14}C]leucine. Concomitant with the shift, the cells were washed, and the medium containing labeled leucine was replaced with the same medium lacking the labeled amino acid. The first dimension was loaded with 10^5 cpm. The numbered proteins are discussed in the text.

laboratories (5, 10, 12). Other metabolic events requiring molecular oxygen might be important to mycelial development. *Saccharomyces* spp. require exogenous sterol and fatty acid when grown anaerobically since molecular oxygen is required for their synthesis. Although *Mucor* spp. grown anaerobically are deficient in both of these classes of lipids (12), we could not demonstrate any effect on morphology by their addition to cultures grown anaerobically (data not shown). In light of our findings with respect to the polypeptides whose synthesis correlates with morphogenesis, we propose that the presence or absence of oxygen controls the induction or repression of polypeptides involved in dimorphism.

The number and pattern of polypeptides detected in this study (about 400) is similar to that reported by Hiatt et al. (4) and Linz and Orlowski (6). In the former study, however, anaerobic (nitrogen-grown) yeasts were not examined, and these were a crucial part of this study as they allow a comparison of the polypeptides synthesized by these cells with those from aerobic mycelia, CO_2-grown yeasts, and most importantly, microaerobic mycelia.

It is apparent that oxygen concentration significantly alters the pattern of polypeptide expression in *M. racemosus*. The response to decreased oxygen is apparently graded. Aerobic mycelial cells when compared with microaerobic mycelia show qualitative and quantitative changes in a relatively large number of polypeptides synthesized. This is consistent with the findings of Hiatt et al. (4) who compared aerobic mycelia with mycelia grown in a standing nitrogen atmosphere (i.e., nitrogen was not continuously sparged through the culture). These changes in polypeptide expression correlate with a change from an oxidative to a fermentative metabolic mode, yet no change in morphology occurs. Yeast morphogenesis only occurs when oxygen is rigorously excluded. When compared with microaerobic mycelia, the yeast morphology of anaerobic cells correlates with alterations in the synthesis of a relatively small number of polypeptides and not with any major change in metabolic pattern or with the levels of glycolytic enzymes (2). This suggests that these polypeptides are involved in the morphogenesis. The fact that the same pattern of changes is seen irrespective of the manner in which yeasts or mycelia are generated (CO_2-grown yeasts and aerobic mycelia also show these changes) strengthens this hypothesis. It appears, therefore, that oxygen controls both the mode of energy metabolism of the cell and morphogenesis but that these are

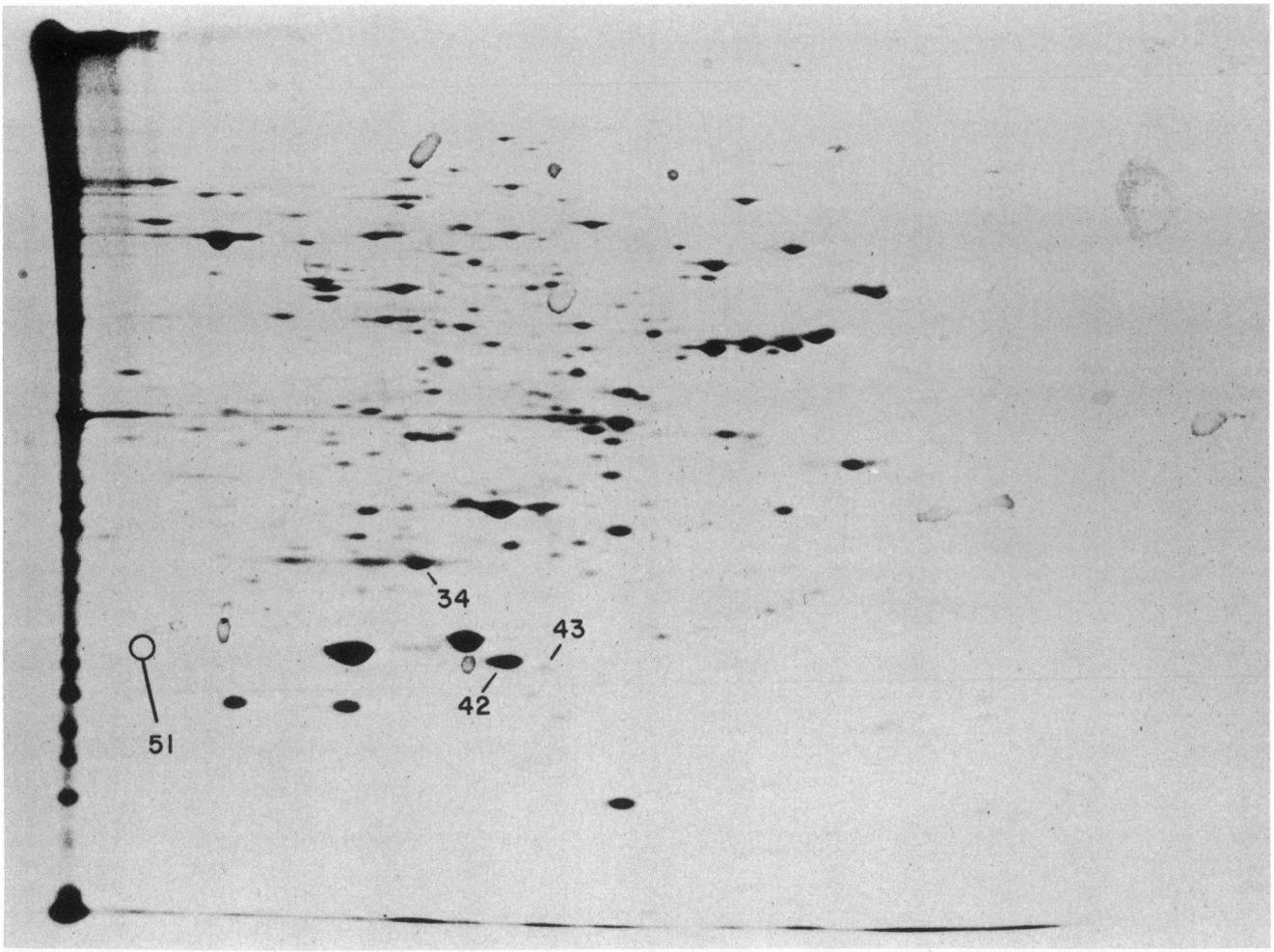

FIG. 9. Autoradiogram of two-dimensional gel of polypeptides from cells 4 h after a shift from anaerobic conditions in an N_2 atmosphere to microaerobic conditions. The preshift culture was labeled with [^{14}C]leucine. Concomitant with the shift, the cells were washed, and the medium containing labeled leucine was replaced with the same medium lacking the labeled amino acid. The first dimension was loaded with 10^5 cpm. The numbered proteins are discussed in the text.

independent events with a common effector. Whether or not these two responses to oxygen are related by common regulatory elements remains to be seen.

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