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# Efficient Sporulation of Yeast in Media Buffered near pH 6

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Diploid cells of *Saccharomyces cerevisiae* underwent meiosis and sporulation when placed in 1% potassium acetate sporulation medium. In unbuffered sporulation medium the pH rose very rapidly, reaching pH 8.4 after 2 h of sporulation. Under these conditions, the uptake of radioactive adenine and lysine was extremely limited, and ascus formation was insensitive to inhibitors such as 5-fluorouracil and canavanine. By using several different buffers, we showed that an increase in the pH of sporulation media was not necessary for sporulation to occur. Spore viability and the kinetics of ascus and prototroph formation were normal for cells sporulated in several types of media buffered as low as pH 5.5. Incubation of sporulating cells below pH 6.5 did cause separation of small but viable buds from their mother cells. With sporulating cells buffered below pH 6.5, the incorporation of radioactive adenine and lysine was greatly enhanced and cells became sensitive to inhibition by 5-fluorouracil and canavanine.

When cells of *Saccharomyces cerevisiae* are placed in acetate sporulation medium, the pH of the medium rises very rapidly from pH 7 to pH 9. This increase occurs regardless of the ability of the cells to undergo meiosis (4), and is thought to be caused either by the respiratory activity of the cells (2) or as a consequence of nitrogen starvation (4). Although the increase in the pH of acetate sporulation medium with time is not a sporulation-specific event, it has been thought to be necessary for sporulation to occur. Mills (6) has reported that buffering acetate sporulation medium below pH 7 inhibits sporulation. However, other experiments by Mills (6) have shown that the pH of sporulation medium may be lowered for a period of 1 h or more with little or no effect on the ability of the cells to complete sporulation.

The high pH of sporulation medium has impeded the study of meiosis in yeast, because the uptake of precursor molecules becomes less and less efficient as the pH increases above 6.0. Uptake of precursor molecules can be made much more efficient by adjusting the pH of the medium down to approximately pH 6 (6). However, this method to increase uptake efficiency also results in an increase in the rates of protein synthesis and ribosomal ribonucleic acid (RNA) processing (12), so that the normal sporulation processes may be temporarily perturbed by the rapid change in the pH of the medium.

To avoid both the effect of high pH on the uptake efficiency of precursor molecules and the cellular response to a sudden adjustment of pH, we looked for conditions under which the entire process of sporulation could occur at a low pH. This would allow the addition of labeled precursor molecules at any point during sporulation without abruptly changing cell physiology by a sudden adjustment of pH. Furthermore, by sporulating cells under pH conditions similar to those found during vegetative growth, it would be possible to distinguish between true sporulation-specific events and nonspecific cell responses to a high-pH acetate sporulation medium.

We have found that yeast cells can sporulate in medium buffered to a low pH and that sporulation under these conditions is normal, as measured by spore viability, prototroph formation, genetic recombination, and ascus formation. Contrary to previous reports (2, 6), an increase in medium pH is not necessary for sporulation to occur. Since sporulation under low-pH conditions can be made efficient, it is now possible to carry out radioisotope labeling and drug inibition experiments which were previously impractical or impossible.

(This work is taken in part from an undergraduate honors thesis submitted by J.M. to the Department of Biology at Brandeis University.)

## MATERIALS AND METHODS

**Yeast strains.** All experiments reported in this paper were performed with *S. cerevisiae* diploid strain AP-1 (obtained from A. K. Hopper). AP-1 was made by crossing two haploid strains: A364A (a *ade1 ade2-1 ura1 gal1 lys2 tyr1 his7*) and $\alpha_1$ 131-20 ($\alpha$ *ade2-2 ura3 can1 cyh2 leu1*). The strain is heteroal-

lelic for *ade2* and has been described previously (4). The markers *gal1*, *lys2*, *tyr1*, and *his7* are on chromosome II, and *ura3* and *can1* are on chromosome V (7).

**Media.** The vegetative pregrowth medium used in these experiments has been described (4, 5, 8, 9). Acetate growth medium (AC) contained 1% potassium acetate, 0.6% yeast nitrogen base, 0.5% yeast extract, 0.5% peptone, and 1.02% potassium biphthalate. The pH was adjusted to 5.5, and 40 μg of adenine and 40 μg of uracil per ml were added to the medium.

Sporulation media contained 1% potassium acetate (1% KAc) as a carbon source. Control cultures were sporulated in unbuffered media that had an initial pH adjusted to either 7 or 5.5. Buffered sporulation media consisted of an 0.2 M solution of one of the following chemicals in 1% KAc: succinic acid (SUC); 2-(*N*-morpholino)ethanesulfonic acid (MES); piperazine-*N*,*N*'bis(2-ethanesulfonic acid) (PIPES); morpholinopropane sulfonic acid (MOPS); or potassium phosphate (PO). SUC and MES media were adjusted to pH 5.5, PIPES medium was adjusted to pH 6.1, MOPS medium was adjusted to pH 6.5, and PO medium was adjusted to pH 7. The pH was adjusted with either HCl or KOH solutions.

Solid agar plates for measuring cell viability contained the following complete medium (COM): 0.67% yeast nitrogen base, 2% glucose, 2% agar, 0.003% leucine, 0.03% threonine, 0.002% tryptophan, 0.002% methionine, 0.002% histidine, 0.002% arginine, 0.003% lysine, 0.002% alanine, 0.002% adenine, 0.0025% uracil, and 0.0025% tyrosine. YEPD plates contained 1% yeast extract, 2% peptone, 2% glucose, and 2% agar. Adenine drop-out plates (ADE), containing everything in COM plates except adenine, were used to measure adenine prototroph formation.

**Growth and sporulation.** Strain AP-1 was pregrown in AC medium supplemented with adenine and uracil. Cells were collected in log phase at a cell density of approximately $3 \times 10^7$ cells/ml. One volume of cell suspension was centrifuged and washed twice with 1 volume of 1% KAc (pH 7). The cells were then suspended in 2 volumes of sporulation medium at a cell density of approximately $1.5 \times 10^7$ cells/ml. Both pregrowth and sporulation cells were aerated at 30°C on a gyratory shaker in flasks with a 1:10 (vol/vol) ratio of liquid to air space.

**Genetic analysis.** After sporulation, the cell walls of asci were digested with the enzyme preparation Glusulase (Endo Labs). Tetrads were then dissected onto YEPD plates as described by Mortimer and Hawthorne (7). After germination and growth, the segregants were picked onto master plates and scored on nutritional dropout plates (10). Dropout plates were essentially COM plates with one amino acid or base left out. Canavanine resistance was scored by replica plating onto arginine dropout plates containing 20 μg of canavanine per ml. Cycloheximide resistance was scored by replica plating to YEPD plates containing 10 μg of cycloheximide per ml. Complementation analysis for *ade1*, *ade2*, *ura3*, and *ura1* was done by crossing the segregants with tester haploid strains and replica plating to minimal media.

**Intragenic recombination.** The procedure of Sherman and Roman (11) was used to detect the adenine prototrophs arising by recombinations between the *ade2* heteroalleles during meiosis. At 1-h intervals, portions of sporulating cells were plated on ADE and COM plates. The number of colonies was counted after 3 to 4 days of growth at 30°C.

**Incorporation of Label.** Sporulating cells were labeled at 6 h into sporulation with 2 μCi of either [$^3$H]lysine or [$^{14}$C]adenine per ml. To measure incorporation of [$^{14}$C]adenine into RNA, duplicate 0.5-ml samples were added to 0.5 ml of cold 10% trichloroacetic acid at different times after the label was first added. After 15 min on ice, the samples were then filtered on 15-mm GF/C filters (Whatman) and washed with cold 5% trichloroacetic acid containing 25 μg of adenine per ml. The filters were dried and the radioactivity was counted in a liquid scintillation counter, using Econofluor as a scintillation cocktail.

Incorporation of [$^3$H]lysine into protein was measured by taking duplicate 0.5-ml samples at intervals after the label was added. The portions were placed in 0.5 ml of 10% trichloroacetic acid and were incubated for 15 min at 90°C. The samples were filtered on GF/C filters and washed with cold 5% trichloroacetic acid. The filters were dried and the radioactivity was counted as described above.

**Isotopes.** [8-$^{14}$C]adenine (7.9 Ci/mol), L-[4,5-$^3$H]lysine (62.25 Ci/mol), and Econofluor were obtained from New England Nuclear Corp., Boston, Mass.

## RESULTS

**Change in pH during sporulation.** The increase in the pH of unbuffered media initially at pH 7 was very rapid, reaching pH 8.4 by about 2 h (Fig. 1). Similarly, cells sporulated in unbuffered 1% KAc with an initial pH of 5.5 were at pH 8.3 after about 4 h. After 8 h, the pH of the unbuffered medium rose very slowly. In contrast to unbuffered sporulation media, the pH of the buffered sporulation media increased very slowly and at a more or less constant rate over a 48-h period (Fig. 1). In all types of unbuffered sporulation medium, the pH increased by only 0.7 pH units over a 48-h period.

**Kinetics of ascospore formation.** The kinetics of ascus formation in buffered and unbuffered sporulation media are displayed in Fig. 2. In the unbuffered pH 7 medium, ascus formation began at 8 h and was essentially complete by 20 h. In the pH 5.5 unbuffered medium, the initial appearance of asci was slightly delayed and was not completed until 36 h; however, the final percentage of asci formed in both experiments was approximately the same (Fig. 2A). The rate of ascus formation in MES, PIPES, and MOPS media (Fig. 2B) as well as the final percentage of sporulation was virtually identical to that of the unbuffered pH 5.5 control. In PO and SUC media, ascus formation occurred at a much slower rate than in either of the

controls (Fig. 2C). In other experiments, spore formation in both PO and SUC media was found to equal and sometimes to slightly exceed that of the controls.

**Separation of mother and daughter cells.** One unexpected finding obtained from this series of experiments was that exposing a log-phase culture of yeast to low-pH sporulation medium resulted in the separation of mother cells from small daughter cells. This separation was measured by plating cells at intervals onto COM plates and counting the colonies after 3 days of growth (Table 1). Whereas some separation occurred in unbuffered pH 7 medium, much more separation was found in unbuffered pH 5.5 medium and buffered medium. More separation was seen in SUC and MES media that remained buffered at pH 5.5 than in the unbuffered pH 5.5 medium.

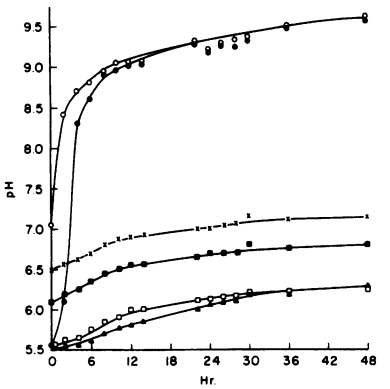


FIG. 1. *Increase in pH in different media during the course of sporulation. Cells were grown to a density of approximately 3 × 10⁷ cells/ml in AC media, washed, and transferred to sporulation media at a density of approximately 1.5 × 10⁷ cells/ml. The pH was determined by taking samples at intervals and measuring the pH on a Radiometer-Copenhagen pH meter. Medium pH over a 48-h period is shown for: pH 7 unbuffered (○), pH 5.5 unbuffered (●), SUC (▲), MES (□), PIPES (■), and MOPS (×) media.*

The separation of mother from daughter cells created a problem in determining the percentage of sporulation. When mother and daughter cells separate, the number of cells per milliliter increases, but small daughter cells generally do not sporulate (3). Counting the separated daughter cells as unsporulated cells would thus decrease the percentage of sporulation of unbuffered pH 5.5 medium and buffered medium relative to unbuffered pH 7 medium. In an effort to avoid this problem, any cell with a diameter less than 75% of that of a mature separated daughter cell was not counted when the percentage of sporulation was determined. In spite of this attempt to correct for the apparent increase in cell number, it is possible that the separation of mother from daughter cells is responsible, in part, for differences in sporulation efficiency and rate of ascus formation in the different media.

**Spore viability.** The viability of spores obtained in different sporulation media was determined by dissecting at least 25 asci in each case. For the pH 7 and pH 5.5 unbuffered controls, approximately 89% of the dissected spores germinated and grew into colonies. Viability in the buffered media ranged from 83 to 89%.

**Intragenic recombination.** AP-1 is auxotrophic for adenine, as it contains two noncomplementing *ade2* mutations. Prototrophs can be generated either through crossing over within the *ade2* locus or by gene conversion. It has been shown that the number of prototrophs increases dramatically during meiosis and that this increase closely parallels meiotic deoxyribonucleic acid (DNA) synthesis (4, 11). Proto-

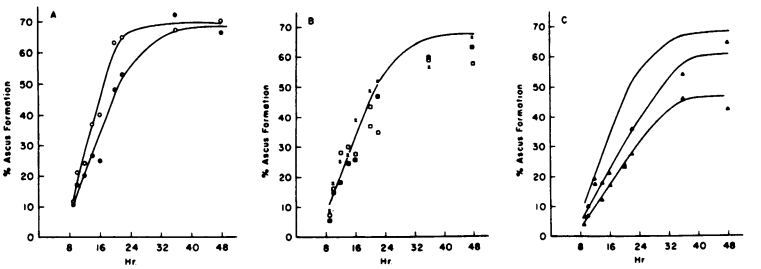


FIG. 2. *Kinetics of ascus formation in different media. Cells were grown to a density of approximately 3 × 10⁷ cells/ml in AC medium, washed, and transferred to sporulation medium at a density of approximately 1.5 × 10⁷ cells/ml. The percentage of ascus formation was determined by examining samples of cells at different times and counting those cells that showed evidence of ascus wall formation as asci. Cells showing no evidence of ascus wall formation were counted as vegetative cells if they had a diameter greater than 75% of that of a mature separated daughter cell. Ascus formation is shown for (A) pH 7 unbuffered (○) and pH 5.5 unbuffered (●) media; (B) MES (■), PIPES (□), and MOPS (×) media; and (C) PO (△) and SUC (▲) media. The line without any points in (B) and (C) represents the rate of ascus formation in unbuffered pH 5.5 medium.*

TABLE 1. *Effect of different media on cell separation*[a]

| Time in sporulation (h) | CFU/ml (× $10^7$) | | | | | | |
|---|---|---|---|---|---|---|---|
| | pH 7[b] | pH 5.5[b] | MES | PIPES | MOPS | PO | SUC |
| 0 | 0.76 | 1.18 | 1.18 | 0.93 | 0.76 | 0.87 | 1.11 |
| 2 | 1.23 | 1.40 | 1.38 | 1.34 | 1.39 | 1.19 | 1.39 |
| 4 | 0.91 | 1.60 | 2.00 | 1.60 | 1.76 | 1.21 | 1.87 |
| 8 | 1.26 | 1.73 | 1.86 | 1.70 | 1.68 | 1.27 | 1.81 |
| 12 | 1.09 | 1.48 | 2.18 | 1.67 | 1.83 | 1.17 | 2.05 |
| 48 | 1.26 | 1.68 | 2.19 | 1.97 | 1.97 | 1.38 | 2.04 |

[a] Cells were grown in AC medium to a density of approximately 3 × $10^7$ cells/ml, washed, and transferred to different types of sporulation media at a cell density of 1.5 × $10^7$ cells/ml. The number of colony-forming units (CFU) per milliliter was determined by diluting samples and plating on COM plates. The plates were incubated at 30°C for 48 h before the number of colonies was determined.

[b] Unbuffered controls.

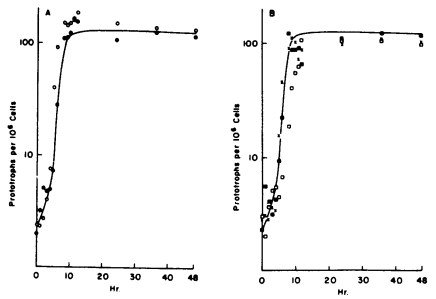


FIG. 3. *Prototroph formation in different media. Cells carrying two ade2 heteroalleles were grown to a density of approximately 3 × $10^7$ cells/ml in AC medium, washed, and transferred to sporulation medium at a density of approximately 1.5 × $10^7$ cells/ml. Prototroph formation was determined by plating samples of cells onto two ADE plates. Adenine-independent colonies were counted after 3 days of growth by dividing the average number of prototrophic colonies per plate by the initial number of cells plated. Prototroph formation is shown for: (A) pH 7 unbuffered (○) and pH 5.5 unbuffered (●) media and (B) MES (■), PIPES (□), and MOPS (×) media. The solid line in (B) represents prototroph formation in pH 5.5 unbuffered medium.*

troph formation was followed by placing samples of cells on ADE plates (Fig. 3).

There was a lag of approximately 1 h in the initial formation of prototrophs in the pH 5.5 unbuffered medium when compared with the pH 7 control (Fig. 3A). This lag increased to approximately 2 h in the later stages of prototroph formation. Each of the two controls produced virtually identical numbers of prototrophs by about 8 h into sporulation.

The final levels of prototrophs formed in MES, PIPES, and MOPS media were very similar and only slightly lower than that of the unbuffered pH 5.5 control (Fig. 3B). The rate of prototroph formation was slightly retarded in MES medium, but was indistinguishable from the pH 5.5 control for PIPES and MOPS. In SUC and PO media, the initial increase in prototroph formation appeared to lag behind the increase in the pH 5.5 control by about 2 h (data not shown). The level of prototroph formation was somewhat lower than that found in MES, MOPS, or PIPES; however, the lower number of prototrophs probably reflects the lower percentage sporulation (Fig. 2C).

**Genetic recombination.** Asci were dissected after sporulating in unbuffered pH 7 medium and in MES and SUC in order to determine the effect of buffers on recombination. The percentage of recombination was measured between four sets of linked markers. Based on approximately 70 tetrads, there was no obvious effect on recombination in the intervals *cyh2-leu1*, *lys2-tyr1*, and *tyr1-his7*. A difference in the percentage of recombination of the *ura3-can1* interval for unbuffered (29.3) and buffered (41.5) media was significant by a contingency $\chi^2$ test ($P < 0.01$); however, the values obtained in the buffered media agreed quite well with the published map distance of about 40 centimorgans (7).

**Uptake of labeled precursors.** The efficiency of uptake of adenine and several amino acids by sporulating cells is greatly influenced by the pH of the medium (6). By lowering the pH during sporulation, the uptake efficiency can be greatly increased. Labeled precursors were added during sporulation to determine how efficiently cells sporulating in buffered medium incorporated [$^3$H]lysine into protein and [$^{14}$C]adenine into RNA as compared with cells in unbuffered medium. The results for lysine incorporation are shown in Fig. 4. As expected, there was little incorporation of label by cells sporulating in unbuffered medium where the pH remained at pH 9. Cells sporulating in buffered medium, however, showed highly efficient incorporation of label. The incorporation

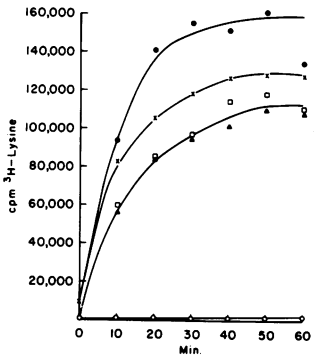


FIG. 4. *Incorporation of [³H]lysine into protein in different media. Cells were grown to a density of approximately 3 × 10⁷ cells/ml in AC medium, washed, and transferred to sporulation medium at a density of approximately 1.5 × 10⁷ cells/ml. Cells were labeled at 6 h during sporulation with 2 μCi of [³H]lysine per ml. The amount of [³H]lysine incorporation was determined from the average hot trichloroacetic acid-precipitable radioactivity from two 0.5-ml samples. The amount of [³H]lysine incorporation is shown for pH 7 unbuffered* (○), *SUC* (▲), *MES* (□), *MOPS* (×), *and pH 7 unbuffered media where the pH was manually adjusted from pH 8.4 to pH 5.8 immediately before adding label* (●).

rates observed in each of the buffered media were almost identical, displaying both similar kinetics and final levels of incorporation. A virtually identical result was found for the incorporation of [¹⁴C]adenine.

**Sensitivity to 5-fluorouracil and canavanine.** Inhibitor studies of sporulation in yeast have been impeded by the insensitivity of sporulating cells to some inhibitors. As a highly potent inhibitor of both DNA and ribosomal RNA synthesis in yeast, 5-fluorouracil is an especially useful drug (1). Cells were sporulated in pH 7 unbuffered medium and MES medium. After 3 h in sporulation medium, by which time premeiotic DNA synthesis has been shown to have begun (4, 11), 5-fluorouracil was added to a concentration of 10, 30, or 50 μg/ml. At such concentrations, 5-fluorouracil has been shown to almost completely inhibit ribosomal RNA synthesis in vegetatively growing yeast in less than 1 h (1). In unbuffered medium there was very little inhibition of sporulation after 40 h (Table 2). However, in medium buffered at pH 5.8 by MES, 5-fluorouracil inhibited sporulation nearly completely.

A similar increase in the effectiveness of canavanine inhibition of sporulation was also found. Canavanine added after 3 h of sporulation at a concentration of 10, 20, or 50 μg/ml did not affect sporulation in pH 7 unbuffered medium, whereas sporulation in MES buffered medium was totally inhibited.

TABLE 2. *Effect of 5-fluorouracil and canavanine on sporulation*[a]

| Medium supplement | Percent sporulation | |
|---|---|---|
| | pH 7 (unbuffered) | MES (buffered) |
| Control | 64 | 67.5 |
| 5-Fluorouracil | | |
| 10 μg/ml | 74 | 6.5 |
| 30 μg/ml | 63.5 | 0 |
| 50 μg/ml | 50.5 | 0 |
| Canavanine | | |
| 10 μg/ml | 82 | 0 |
| 20 μg/ml | 71 | 0 |
| 50 μg/ml | 80 | 0 |

[a] Cells were grown in AC medium to a density of approximately 3 × 10⁷ cells/ml, washed, and transferred to pH 7 unbuffered medium and pH 5.5 MES medium at a cell density of approximately 1.5 × 10⁷ cells/ml. After 3 h in sporulation medium, either 5-fluorouracil or canavanine was added at different concentrations. Sporulation efficiency was measured after 40 h. Those cells with a diameter less than 75% of that of a mature, separated daughter cell were not counted.

## DISCUSSION

We have found that *S. cerevisiae* diploids can sporulate in 1% KAc buffered between pH 5.5 and 7, in contrast to previous reports that sporulation was severely inhibited below pH 7 (2, 6). In general, sporulation is most efficient with buffers that cannot be metabolized, such as MES, MOPS, and PIPES, but even PO buffer will support sporulation.

One of the major limitations in studying the metabolism of macromolecules, especially DNA and RNA, during meiosis and sporulation of yeast has been the problem of incorporation of labeled precursors. We have found that a number of buffers can be used to maintain sporulating cells at pH conditions that are similar to those found in vegetative growth but still allow efficient sporulation. In particular, the buffers (MES, MOPS, and PIPES), which probably are not metabolized by the cells, support sporulation and genetic recombination only slightly less efficiently than do unbuffered controls. The maintenance of sporulating cells at a constant pH below pH 7 permits isotopic labeling or the addition of inhibitors such as 5-fluorouracil or canavanine without subjecting cells to an abrupt pH shift.

We had previously found that shifting sporulating cells in unbuffered medium from pH 9 to pH 7 resulted in a twofold increase in the rate of protein synthesis and in the processing of ribosomal RNA (12). In this study, we have shown that maintaining cells at a low pH during the

entire course of sporulation does not seem to accelerate the appearance of mature asci or the increase in the genetic recombination. It may be that the changes in biosynthesis observed soon after lowering the pH are transient, since the major landmark events associated with meiosis and sporulation are not affected by sporulating cells in buffered media.

The only significant change in yeast cells sporulating at lower pH is that small buds detach from mother cells. These small cells are viable but do not sporulate, probably because the cells are too small to support the turnover and utilization of stored macromolecules necessary for sporulation. The effect of these small cells on measurements of macromolecular synthesis is probably not different from conditions under which these cells remain attached to the mother cell.

A detailed study of meiosis in yeast depends in large measure on being able to incorporate heavy isotopes or radioactive precursors of DNA. Similarly, labeling of RNA for short periods in buffered media permits the isolation of messenger RNA of high enough specific activity to measure messenger half-life and related values. Such experiments should now be possible.

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