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Macromolecule Synthesis and Breakdown in Relation to Sporulation and Meiosis in Yeast

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The time course of synthesis and breakdown of various macromolecules has been compared for sporulating (a/α) and nonsporulating (a/a and α/α) yeast cells transferred to potassium acetate sporulation medium. Both types of cells incorporate label into ribonucleic acid and protein. The gel electrophoresis patterns of proteins synthesized in sporulation medium are identical for sporulating and nonsporulating diploids; both are different from electropherograms of vegetative cells. Sporulating and nonsporulating strains differ with respect to deoxyribonucleic acid synthesis; no deoxyribonucleic acid is synthesized in the latter case, whereas the deoxyribonucleic acid complement is doubled in the former. Glycogen breakdown occurs only in sporulating strains. Breakdown of preexisting vegetative ribonucleic acid and protein molecules occurs much more extensively in sporulating than in nonsporulating cells. A timetable of these data is presented.

During ascospore development, yeast cells undergo both physiological change, from vegetative growth through spore morphogenesis to dormancy, and a change in genetic constitution, from diploid through meiosis to haploid. To initiate this complex series of events, both genetic and physiological signals are required. Only diploid cells heterozygous (a/α) at the mating type locus are able to undergo premeiotic deoxyribonucleic acid (DNA) synthesis (33), meiotic recombination (12, 32), and sporulation (30). Diploid cells homozygous (a/a or α/α) for mating type are not capable of these processes (12, 30, 34). A state of cellular metabolism conducive to sporulation can be brought about by nitrogen starvation with good aeration and a carbon source other than glucose or ethanol (11, 23).

In the pioneering biochemical and physiological studies of Croes (3, 4), sporulation was obtained by exposure of cells to potassium acetate after vegetative growth in complete medium containing glucose. During the subsequent development of asci, Croes observed and quantitated a sequence of biochemical changes in the yeast cells. Among these were: (i) a 1.5- to 2-fold increase in DNA content; (ii) a 1.46-fold increase in dry weight, largely accounted for by increases in lipid and carbohydrate; (iii) a decrease in ribonucleic acid (RNA) content (to 0.5 the initial value) and a slight decrease in protein; (iv) a large increase in O_2 consumption by the culture, reaching a maximum at 2 h after the shift to sporulation medium, with a subsequent steady decline; and (v) a rise in medium pH from 7 to 9.5.

Observations ii, iv, and subsequent metabolic studies indicated that acetate from the medium is both oxidized by the cells in energy-yielding reactions (8) and converted to storage carbohydrate via gluconeogenesis (31).

In the sporulation regimen used by Croes, aerobic respiratory adaptation began at the end of vegetative growth on glucose medium and was completed upon the shift to acetate sporulation medium. That respiration is necessary for sporulation is indicated by the fact that respiratory-deficient yeast mutants cannot sporulate (6, 36). Consequently, certain early biochemical changes observed in such cultures may pertain only to respiratory adaptation and not to sporulation.

Roth and Halvorson (33) succeeded in dissociating respiratory adaptation from sporulation with acetate as the carbon source in the presporulation growth medium. The kinetics of ascospore formation were demonstrated to be both more rapid and more synchronous in such acetate-pregrown cultures than in cultures shifted from glucose growth medium to acetate sporulation medium.

Using the Roth and Halvorson respiratory

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preadaptation procedure, we have studied biochemical changes accompaning sporulation. Our intent was to determine the temporal order of the various events in the acetate → acetate shift. Similar data on the order of events in glucose to acetate cultures have been assembled (1, 4, 8, 39). We have performed all measurements (except protein and RNA breakdown) on a single culture. Such an approach allows one not only to fix firmly the order of events, but also to separate events close in time, such as DNA synthesis and nuclear division.

With a few exceptions (1, 12, 32, 34), no separation has been made of events specific to sporulation from events which are a generalized response to the stepped-down culture conditions. When nonsporulating diploids are used as controls, it is important to ascertain the cause of their failure to sporulate. We have determined the degree of correlation between each biochemical event and sporulation by carrying out measurements on cultures of both sporulating (a/α) and otherwise isogenic (a/a or α/α) diploids homozygous for mating type.

MATERIALS AND METHODS

Yeast strains. All yeast strains used in this work were derived from two haploids: A 364A (*a ade1 ade2 ura1 his7 lys2 try1 gal1;* [15]) and α_1 131-20 (α *ade2* [noncomplementary with *ade2* from A 364A] *ura3 cyh2 can1 leu1*). Genetic nomenclature is as described by Mortimer and Hawthorne (26). The α strain is a haploid derived by sporulation of diploid a/α strain 131-20 (37). Our standard a/α strain, AP-1, was made by crossing A 364A with α_1 131-20. Corresponding a/a and α/α diploids were derived from AP-1 by 1 min of ultraviolet irradiation (approximately 18.4 ergs/mm^2 per s of 1 ml of cells at 1×10^8 cells per ml of water to induce mitotic recombination (29) leading to homozygosity at the mating type locus. Mitotic recombinants which possessed either an a or α mating type were scored by their ability to mate with haploid strains 56a or S2072α (obtained from L. Hartwell), respectively, to yield *ADE2* zygotes. AP-1-a/a and AP-1-α/α clones tested positive for mating response, inability to sporulate at 24 h, and segregation of asci containing four a/α spores after mating with known a/a or α/α diploids. However, it was found that these strains were capable of forming a small percentage of asci (0.05% for AP-1-α/α and 1% for AP-1-a/a) after 3 days on sporulation medium. Similar results for other a/a and α/α strains have been reported (40). This low efficiency sporulation probably represents "leakiness" of mating type control over sporulation, but does not negate the use of a/a and α/α homozygous mating type diploid cells as nonsporulating controls. These strains will be described elsewhere (A. K. Hopper and B. D. Hall, manuscript in preparation).

Growth and sporulation of yeast. The yeast strains were pregrown in acetate medium as described by Roth and Halvorson (33) with the exception that 40 μg of adenine and uracil were added per ml of growth medium. After 30 h of continuous exponential growth, the cells (1.5×10^7 to 2.0×10^7 per ml) were quickly filtered into a membrane filter (Millipore Corp.), were washed with approximately a 0.5 volume of sporulation medium, and were suspended into sporulation medium (0.3% potassium acetate, 0.02% raffinose) (33) at the same cell density.

Respiration. Oxygen consumption was measured by a YS1 model 53 biological oxygen monitor.

DNA synthesis. Cellular DNA content was measured by the diaminobenzoic acid fluorimetric procedure of Kissane and Robins (18) modified for yeast analysis by C. Milne (Master thesis, University of Washington, Seattle, 1972). For each time point, triplicate 1-ml (1.5×10^7 to 2.0×10^7 cells per ml) samples were hydrolyzed in 1 N NaOH for 24 h at room temperature. The hydrolyzed samples were chilled, cold trichloroacetic acid was precipitated, and the samples were collected at 4 C by centrifugation at $1,400 \times g$ for 20 min and subsequently were washed with 1 volume of 5% trichloroacetic acid, 0.1 M potassium acetate in 95% ETOH, and 100% ETOH. The washed pellet was dried at 60 C. The dried pellet was reacted with 62 mg of decolorized (by Norit) diaminobenzoic acid in 100 μliters of water for 30 min at 60 C followed by the addition of 2.0 ml of 0.6 N $HClO_4$ to the DABA-DNA solution. The fluorescence (excitation at 408 nm and emission at 508 nm) was determined on an Aminco-Bowman spectrophotofluorimeter.

Nuclear division. Nuclei were stained after fixation with 4% formalin-saline by the method of Robinow and Marak (28), as modified by Hartwell (16).

Intragenic recombination. Recombination between the two noncomplementary *ade2* alleles was followed by scoring for adenine prototrophs by the procedure of Sherman and Roman (35). At 2-h intervals, two 0.1-ml samples of cells in sporulation medium were spread onto two plates lacking adenine but containing all other required nutrients. The number of recombinant colonies per plate was determined after 2 days of incubation at room temperature.

Measurement of protein and RNA synthesis. Samples (1.5 ml) were removed from the sporulating culture at intervals and filtered on membrane filters (Millipore Corp.), and the filter was suspended in 1.5 ml of sporulation medium adjusted to pH 6.0, containing 1.5 μCi of [^{3}H]adenine (50 μCi/μmol) and 0.75 μCi of [^{35}S]methionine (3.75 mCi/μmol for a/α cells and 0.75 mCi/μmol for a/a cells). After 10 min of incubation with shaking at 30 C, duplicate 0.5-ml portions were pipetted into 0.5 ml of 10% trichloroacetic acid containing cold methionine and adenine. One tube was heated at 90 C for 15 min; the other was kept on ice. The cells were collected on Schleicher and Schuell Inc. glass filters, washed with 5% trichloroacetic acid containing methionine and adenine, and counted in a liquid scintillation counter. Hot trichloroacetic acid-insoluble ^{35}S radioactivity was considered a measure of protein synthesis; cold minus hot trichloroacetic acid-insoluble ^{3}H radioactivity (corrected for ^{35}S spillover) was considered a measure of RNA synthesis.

Measurements of soluble pools. Portions of sepa-

rate cultures labeled for six generations during vegetative growth with [^{3}H]adenine or [^{35}S]methionine were filtered and suspended in equal volumes of sporulation medium (pH 6.0) containing [^{14}C]adenine or [^{3}H]methionine. After 10 min, the cells were filtered on a membrane filter (Millipore Corp.) which was placed into a test tube to which cold trichloroacetic acid was added. After centrifugation the trichloroacetic acid supernatant was spotted on a filter paper disk (Whatman 3 MM) and counted. The ratios of [^{14}C]adenine to [^{3}H]adenine or [^{3}H]methionine to [^{35}S]methionine were taken as a measure of the specific activity of the pulse-labeling isotope at the end of the labeling period.

Polyacrylamide gel electrophoresis. Slab sodium dodecyl sulfate-polyacrylamide gel apparatus and procedure were those of Studier (38). The sodium dodecyl sulfate gels, buffer system, and sample preparation were those of Laemmli (19).

Lysates were prepared by homogenizing the following mixture for 15 s in a Braun homogenizer: 0.2 ml of cells at 0.5×10^9 to 1.0×10^9 cells per ml suspended in 0.0625 M tris(hydroxymethyl)aminomethane (pH 6.8), 5% mercaptoethanol, 3% sodium dodecyl sulfate, 0.3 mg of phenylmethylsulfonylfluoride (protease inhibitor; Sigma) per ml, and 0.5 ml of glass beads (0.45 mm diameter). Cell lysates were eluted from the glass beads with three 0.2-ml buffer-phenylmethylsulfonylfluoride rinses, immediately boiled for 3 min, and frozen at −30 C. Between 5 and 40 μliters of each prepared lysate was loaded into the sample gel well for electrophoresis.

Protein and RNA breakdown. Protein and RNA breakdown were measured as the loss of vegetatively labeled intracellular macromolecules. Growing yeast cells were simultaneously labeled with 2μCi of [^{3}H]adenine per ml (3.0 Ci/mmol) and 2.7 μCi of [^{35}S]methionine per ml (34 Ci/mmol) for six generations prior to sporulation. The cells were washed free of radioactive medium and placed in sporulation medium. At the initiation of sporulation and at 1- to 2-h intervals, two 0.5-ml portions of the culture were removed and centrifuged at $2,000 \times g$ for 3 min. The supernatant (20 μliters) was pipetted onto glass-fiber filters, the pelleted cells were resuspended by vortexing, and 0.5 ml of 10% trichloroacetic acid was added to each sample. One sample (hot trichloroacetic acid) was hydrolyzed at 95 C for 15 min and the other (cold trichloracetic acid) was chilled. The hot and cold trichloracetic samples (at 4 C) were filtered onto glass-fiber filters, washed, dried, and counted. RNA was taken as the cold minus hot acid-precipitable ^{3}H radioactivity. Protein was taken as hot or cold acid-stable ^{35}S radioactivity. RNA and protein breakdown were determined either by the loss from the acid-precipitable fraction of vegetatively labeled RNA and protein or by the appearance of acid-soluble radioactivity in the medium used for sporulation.

Thin-layer chromatography. Thin-layer chromatography was performed with methanol-washed (one time) polygram cell 300 polyethylenemine cellulose plates (20 by 20 cm). Nucleotides present in a concentrated portion of medium were chromatographed in 0.5 M formate (to 0.5 of the height of the chromatogram) followed by chromatography in 2 M formate. Marker migration was determined by ultraviolet adsorption.

Glycogen content. Glycogen synthesis and breakdown were monitored by a procedure devised by G. Whelan, University of Miami Medical School, Miami, Fla. (personal communication to M. Friedman). At 2-h intervals after sporulation, two 10-ml portions of cells were centrifuged at approximately $2,500 \times g$ for 3 min and frozen. The redissolved pellet was hydrolyzed in 1 ml of 20% KOH for 1 h at 100 C and neutralized with 1 ml of 3.5 N HCl, and the glycogen was precipitated with 2 volumes of 95% ETOH. The precipitate was collected by low-speed centrifugation, washed twice with 66% ETOH, oven-dried at 65 C, and then suspended in 1.8 ml of 50 mM acetate buffer (pH 5.0) with sonic treatment. Glycogen was degraded to glucose by incubation with 0.2 ml of amyloglucosidase (5.9 mg/ml) [isolated from *Aspergillus* (Miles) by M. Friedman] at 35 C for 2 h. The resulting glucose was assayed by the glucose oxidase assay of Lee et al. (20).

RESULTS

For all experiments, strains AP-1-a/α and otherwise isogenic AP-1-a/a and AP-1-α/α were maintained in log-phase growth for 30 h on acetate medium (33). At a density of 1.5×10^7 to 2.0×10^7 per ml, cells of each genotype were transferred to sporulation medium, and samples of a principal culture of each were removed to measure respiration, pH change, ascus formation, DNA synthesis, nuclear division, recombination, protein, RNA, and glycogen synthesis and breakdown. These experiments were repeated four times with consistent results. The midpoint of a particular transition or event was constant to within 1 h from one experiment to another. The timing of events relative to one another was also reproducible, except for a considerable variation in the time at which protein and RNA synthesis were shut off. A time scale of this relationship is given in Fig. 7.

Respiration, pH, and ascus formation. The relationship between change in O_2 consumption, increase in pH, and ascus formation is given in Fig. 1. There is an initial increase of 10 to 20% in oxygen consumption at 2 h into sporulation, followed by a steady decline. This pattern of respiration agrees qualitatively with previously reported data (3). However, we did not see the dramatic early increase in oxygen consumption which Cores reported, probably because our cells are preadapted to aerobic growth on acetate medium. The respiration curves obtained for a/a and α/α diploids in sporulation medium were indistinguishable from those of a/α cells.

The increase in medium pH from 6.7 to between 8.5 and 9.0 agrees well with previously

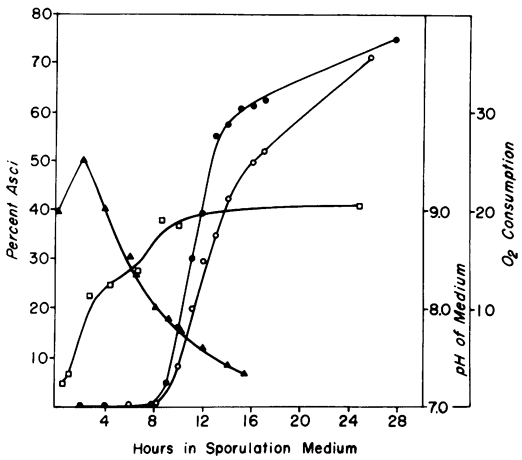

FIG. 1. *Temporal relationship between O_2 consumption, increase in medium pH, and ascus formation. Cultures of AP-1-a/α cells were pregrown to 2.0 × 10⁷ cells per ml in presporulation medium, filtered, washed, and transferred to sporulation medium at 2.0 × 10⁷ cells per ml. At 2-h intervals 3.0 ml of cells were removed to measure the percentage of oxygen consumed per minute (▲) with an oxygen probe. Oxygen consumption curves of cells in sporulation medium were identical for a/α, a/a, and α/α cultures. At similar times a 5.0-ml portion of cells was removed, and the pH of the medium was determined (□). pH curves were identical for a/α, a/a, and α/α cultures. At 1-h intervals 0.1-ml portions of cells were removed and fixed with 0.1 ml of 8% formalin-saline. The percentage of cells as asci was determined by light microscopy (●, ○; two independent experiments); respiration and pH for both experiments were identical. At 28 h no asci were detectable in a/a or α/α cultures.*

reported data (3, 13, 24). Again, sporulating and nonsporulating diploids in sporulation medium give the same result.

The time course of ascus formation observed in two separate experiments with the same a/α diploid yeast strains is shown in Fig. 1. In the period between 9 to 10 and 14 to 16 h, more than 50% of the cells form asci. An additional 15 to 20% of the cells complete ascus formation within a subsequent 24-h period. A large proportion of these late developing asci occur in small buds attached to mother cells which have already formed asci.

DNA synthesis, nuclear division, and intragenic recombination. The kinetics of DNA synthesis during sporulation, followed by fluorimetric measurements (Fig. 2), agrees with the results of Roth and Lusnak (34). Whereas a/α cells increase their DNA nearly twofold on transfer to sporulation medium, a/a and α/α cells show less than a 10% increase in DNA under these conditions. DNA synthesis in a/α cells beings at 2 h and is largely completed by 8 h. This time spread and the subsequent slight DNA increase from 8 to 24 h probably reflect asynchrony of the cell population with respect to initiation of sporulation.

Although meiosis in yeast occurs without dissolution of the nuclear membrane (25), meiosis I and II can be observed by the segregation of Giemsa-staining material to opposite poles of the nucleus (27). We refer to binucleate and tetranucleate cells in describing nuclei which have undergone meiosis I and II, respectively, although these terms are misnomers in a strictly cytological sense. Figure 2A shows the percentage of binucleate cells as a function of time in sporulation medium. The first binucleate cells appear at 4 h, about 1.5 h after the first measured increase in DNA content. The maximum number of binucleate cells, 20%, is found at 6 h, after which the number declines as tetranucleate cells begin to appear. (We have not distinguished between tetranucleate cells

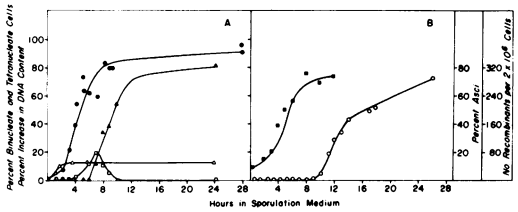

FIG. 2. *Temporal relationship between DNA content, recombination, binucleate and tetranucleate cells, and ascus appearance. The cell culture was the same as in the legend to Fig. 1. At 1-h intervals, two 1-ml portions were removed and hydrolyzed in 1.0 N NaOH for 24 h at 23 C, acid-precipitates were reacted and hydrolyzed with 2,3-diaminobenzoic acid, and fluorescence was determined. The calculated percentage of increase in fluorescence with time was taken to be the increase in cellular DNA content (●), (△) for a/α and α/α cells, respectively. Intragenic recombination was scored as the number of adenine prototrophs appearing during sporulation. Binucleate and tetranucleate cells were scored by the Giemsa-staining technique. At 1-h intervals 0.1-ml portions of cells were removed from the parent culture and fixed with 0.9 ml of formalin-saline. The cells were fixed to slides and stained, and the percentage of cells appearing as binucleate (□) and tetranucleate (▲) were scored. No discrimination between tetranucleate cells and asci was made. At 1-h intervals two 0.1-ml portions of the culture (equal to 2 × 10⁶ cells) were spread onto two nutrient plates lacking adenine. The resulting average number of colonies per plate was taken as the number of intragenic recombinants per 2 × 10⁶ cells (■). The percentage of the cells as asci (○) is as in the legend to Fig. 1. None of the above events occurred in a/a or α/α cells.*

and asci in these counts.) The final percentage of tetranucleate cells is only about 5% greater than the final percentage of sporulation, indicating that nearly all cells which reach the tetranucleate stage can go on to form asci recognizable in the light microscope.

Intragenic recombination can be scored in our strain, which is heterozygous for two noncomplementary mutant alleles at the *ade2* locus. The percentage of adenine prototrophs in the population at a given time is a measure of the number of recombination events which have taken place between the mutant sites (35). Figure 2B shows the appearance of adenine prototrophs during sporulation. The rise appears to be continuous from 2 to 9 h, at which point the number levels off and remains constant.

Total protein and RNA synthesis. Yeast cells in sporulation medium were simultaneously pulse-labeled with [^{3}H]adenine and [^{35}S]methionine to determine the relative rates of synthesis of RNA and protein at different times. Labeling was carried out in fresh sporulation medium adjusted to pH 6.0. Previous work by Mills (24) showed that the high pH (6.7 to 9.0) reached in sporulation medium inhibits uptake of RNA and protein precursors. RNA and protein labeling in 10-min pulse-incorporation experiments performed at 1-h intervals during sporulation are shown in Fig. 3. The incorporation profile shows substantial incorporation into protein between 2 and 6 to 8 h. In some experiments these periods of incorporation show up as separate peaks on a time curve; in others, as in Fig. 3, there is a single broad peak. Although the maximum in the RNA-incorporation curve varied between 5 and 6 h into sporulation, a 2- to 3-h lag of the peak of RNA synthesis behind the peak of protein synthesis (Fig. 3A) was observed in all experiments. Because protein and RNA synthesis are measured by simultaneous incorporation of [^{3}H]adenine and [^{35}S]methionine into the same culture portion, the separation in time between the two processes is not caused by a physiological variation between different yeast cultures. Moreover, the lag in RNA labeling appears not to be caused by variations with time in relative labeling of the RNA and protein precursor pools, since the specific activities of protein and RNA pool precursors remained constant throughout sporulation.

In *a/a* and α/α diploid strains (Fig. 3B, data not shown for α/α) maximal incorporation into RNA and protein is observed at roughly the same times as in *a/a* cells. Although the protein

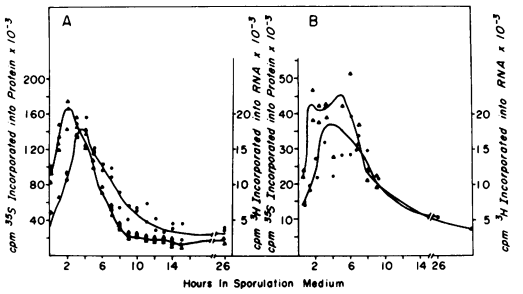

FIG. 3. *Protein and RNA synthesis in a/α (A) and α/α (B) cells. Cell cultures were the same as in the legend to Fig. 1. At 1-h intervals two 1.5-ml portions were filtered, suspended in potassium acetate labeling medium (pH 6.0), and pulse-labeled. The acid-precipitated samples were filtered, and the average counts per minute incorporated into protein (▲, hot acid-precipitable ^{35}S counts per minute) and RNA (●, cold minus hot acid-precipitable ^{3}H counts per minute) were determined.*

synthesis curve shown for *a/a* (Fig. 3B) appears broader than that for *a/α* (Fig. 3A), the differences between them are no greater than the variations between replicate experiments with the same strain.

Synthesis of new proteins. The observations that both heterozygous (*a/α*) and homozygous mating type (*a/a*, α/α) diploid cells synthesize proteins in sporulation medium raised a question as to whether the same proteins were being synthesized in each case. Proteins extracted from *a/α* and α/α cells pulse-labeled for 10 min with [^{35}S]methionine or [^{14}C]leucine either during vegetative growth or at 2-h intervals after transfer to sporulation medium were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Both the pattern and relative intensity of stainable protein bands are identical for all cells at all sampling times. However, the autoradiogram patterns (newly synthesized proteins) of vegetative cells differ from those obtained from cells incubated in sporulation medium. Figure 4A and B shows the autoradiogram pattern of labeled vegetative log proteins (no. 17 on Fig. 4A; no. 1, 3, and 4 on 4B) and the protein pattern from *a/α* cells 6 h after transfer to sporulation medium (no. 18 on Fig. 4A). Many vegetative proteins are not synthesized during sporulation and, conversely, a number of new protein bands appear. The change from a vegetative pattern is later than 15 min (no. 2–5 on Fig. 4A) and prior to 2 h after the shift to sporulation medium; the new pattern persists for as long as appreciable protein synthesis continues in sporulation medium (up to 12 h).

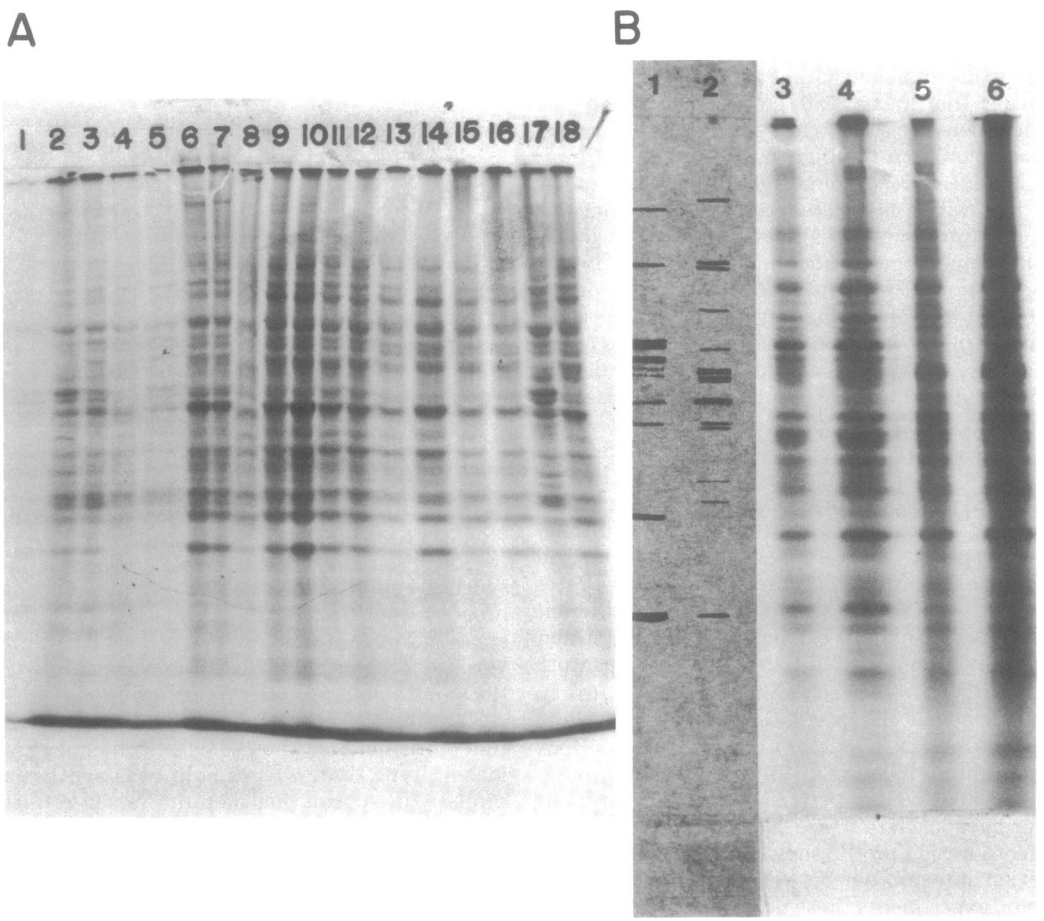

FIG. 4. *(A) Autoradiogram of pulse-labeled proteins of a/α, α/α, a, and α cells in vegetative and sporulation medium. Cells were grown as previously described. Prior to transfer to sporulation medium a 10-ml portion of cells was labeled with 1 μCi of [^{35}S]methionine per ml (3.75 Ci/mmol) for 5 min. At 2-h intervals 10-ml portions of cells in sporulation medium were pulse-labeled for 5 min at pH 6.0 with the above [^{35}S]methionine. At the end of the pulse, cells were centrifuged and frozen until prepared for electrophoresis. The prepared protein samples were subjected to electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide slab gel at 30 V for 15 h. The gel was stained, dried, placed on X-ray film, and exposed for 4 days. Samples on gel and resulting autoradiogram were as follows: 1, a/α cells in vegetative medium: 2–5, a/α, α/α, α, and a cells, respectively, pulse-labeled after 15 min in sporulation medium; 6–8, a/α, α/α, and α cells, respectively, pulse-labeled after 2 h in sporulation medium; 9–12, a/α, α/α, α, and a cells after 6 h in sporulation medium; 13–16, a/α, α/α, α, and a cells after 10 h in sporulation medium; 17, 1 repeated; 18, 9 repeated. (B) Autoradiogram of pulse-labeled proteins of a/α cells in vegetative and sporulation media. 1, Simulation of bands appearing in greater intensity or present in 3 and 4 but not in 5 and 6; 2, simulation of bands appearing in greater intensity or present in 5 and 6 but not in 3 and 4; 3, 4, a/α cells in vegetative medium; 5, 6, a/α cells pulse-labeled after 4 h in sporulation medium.*

The most striking observation is that the same patterns of proteins newly synthesized in sporulation medium are obtained for a/α cells capable of sporulation and for α/α, a, and α (no. 6–16 on Fig. 4A) cells which fail to undergo sporulation. No protein bands have been identified as being specific to a/α cells.

Protein and RNA breakdown. Measurements of protein and RNA breakdown were performed on cells labeled for six generations in vegetative medium with both [^{3}H]adenine and [^{35}S]methionine. Greater than 90% of the cold acid-precipitable ^{3}H radioactivity present at the end of labeling was ribonuclease sensitive. Labeled cells were transferred to sporulation medium, and portions were removed at 1-h intervals for analysis. Protein and RNA breakdown were determined by both the decrease in acid-precipitable radioactivity and by the amount of radioactivity released into the me-

dium. There are three salient features of these analyses (Fig. 5). (i) RNA and protein breakdown begins immediately after the cells are placed in sporulation medium and continues at the same rate until approximately the time asci first appear. (ii) All RNA and protein radioactivity which disappears from the intracellular acid-precipitable fraction is recovered as extracellular acid-soluble radioactivity. The labeled adenine in this fraction was shown by thin-layer chromatography to be present in 2'- and 3'-adenosine 5'-monophosphate. Control cultures, similarly prelabeled and allowed to continue growth in vegetative medium, released no radioactivity into the culture medium. (iii) By the time sporulation is substantially completed in a/α cells (24 h), 50 to 70% of the total preexisting RNA is broken down in a/α cells and 20 to 30% is broken down in a/a and α/α cells. Protein breakdown proceeds to the extent of 25 to 30% of the initial amount in a/α and proceeds to 0 to 10% in a/a or α/α cells. These values represent minimal estimates, as they are derived from net protein and RNA and do not take into account reutilization of breakdown products for new protein and RNA.

Glycogen synthesis and breakdown. Figure 6A shows the change in glycogen content in a/α cells as a function of time in sporulation medium. The cells contain very little glycogen (less than 4 μg of glycogen per ml) at the time they are shifted from vegetative to sporulation medium. Net glycogen synthesis begins between 0 and 2 h and continues until about 8 h into sporulation, with the maximal glycogen content reaching about 60 to 80 μg of glycogen per ml of cells. Figure 6B shows that a/a cells also synthesize glycogen with about the same kinetics as a/α cells. Therefore glycogen accumulation, like protein and RNA synthesis, appears to be primarily a response to the shift to sporulation medium, rather than a sporulation-specific process. Glycogen breakdown in a/α cells, but not in a/a or α/α cells, has been previously reported (17; S. M. Kane, R. M. Roth, and J. A. Erwin, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 184, 1972). In a/α cells glycogen begins to break down about 8 h into sporulation, decreasing rapidly until about 12 h, after which it continues to decrease more slowly. These results confirm those of Kane et al. (17; Kane, Roth, Erwin, Abstr. Annu. Meet. Amer. Soc. Microbiol., 1972), who found similar kinetics of glycogen accumulation. They report considerably higher accumulation, with the maximum at 0.7 mg per 10^8 cells, whereas our values correspond to 0.26 mg per 10^8 cells. They also report glycogen breakdown in a/α cells such that at the end of sporulation the level is 75% of the maximum, whereas in our strain it is 25%.

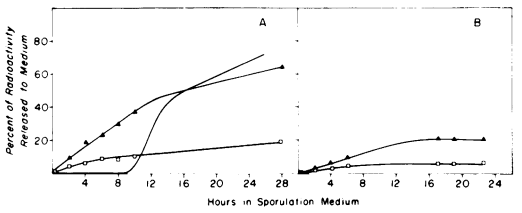

FIG. 5. *Breakdown of RNA and protein in a/α (A) and a/a (B) cells in sporulation medium. Cell cultures were grown as previously described. Six generations prior to sporulation 2 μCi of [^{3}H]adenine per ml (3.0 Ci/mmol) and 2.75 μCi [^{35}S]methionine per ml (34 Ci/mmol) were added to the vegetative medium. At 2.0 × 10^7 cells per ml the cultures were filtered, washed, and suspended in sporulation medium. The amount of radioactive precursor in high-molecular-weight RNA and protein was determined. At 2-h intervals two 1-ml portions of cells were centrifuged, 20 μliters of the cell medium was spotted onto S & S glass-fiber filters, and the radioactivity was determined. The average radioactivity of the two samples in the cell medium was used to determine the percentage of macromolecular breakdown and release to the cell medium. Symbols: ▲, percentage of [^{3}H]adenine or RNA broken down and released; □, percentage of [^{35}S]methionine or protein broken down and released. Ascus formation (——) is as in Fig. 2.*

DISCUSSION

One purpose of this work was to determine the temporal order of the various sporulation-specific events using the acetate-pregrowth sporulation procedure. Such a timetable is shown in Fig. 7. The events expected to be transitory (premeiotic DNA synthesis, meiotic recombination, and presence of binucleate

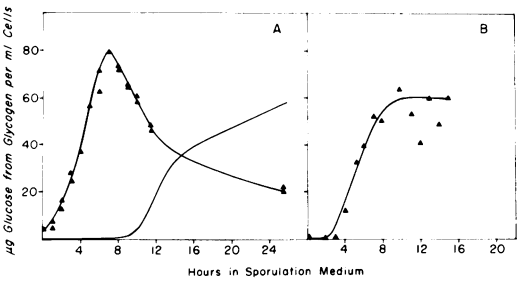

FIG. 6. *Glycogen synthesis and breakdown in a/α (A) and a/a (B) cells in sporulation medium. Two 5-ml portions each of a/α and a/a cells (same cultures as described in the legend to Fig. 1) were centrifuged and frozen. Glycogen was precipitated and enzymatically broken down to glucose, and the resulting micrograms of glucose per milliliter of cells (▲) was determined by the glucose oxidase method. Ascus formation (——) is as in Fig. 2.*

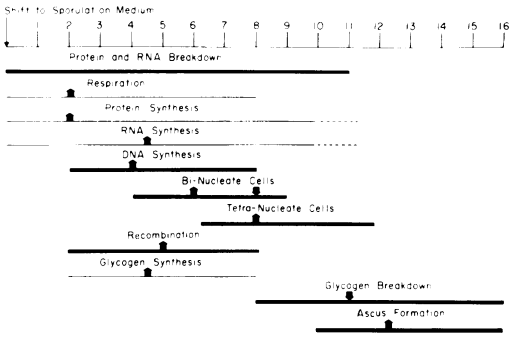

FIG. 7. *Temporal relationship of events monitored for a/α, a/a, and α/α cells in sporulation medium. A composite of Fig. 1–3, 5, and 6 is given here. Numbers in the top line represent hours in sporulation medium. (——), Events unique or partially unique to cells capable of ascus formation; (——), events occurring in cells capable of ascus formation (a/α) as well as those incapable (a/a, α/α) of sporulation. Lines begin at times when events are first detectable and terminate when events are no longer detectable or when a plateau is reached. (↑), Peak of oxygen consumption, protein and RNA synthesis, the midpoint of increase in DNA content, the percentage of binucleate and tetranucleate cells, gene conversion, glycogen synthesis, and ascus formation; (↓), midpoint of decrease of the percentage of binucleate cells and glycogen breakdown.*

cells) all span considerable time periods. The length of each such time span is partially due to asynchrony between cells in sporulating population, which we estimate to be from 2.5 to 5 h.

No major discrepancies are apparent between our timetable for biochemical events during the sporulation of acetate-grown cells and the timetable constructed by Tingle et al. (39) from results obtained in a number of laboratories on glucose → acetate-shifted cultures. It is of interest to compare the timing of some sporulation-specific events. DNA synthesis in the glucose → acetate regimen requires 6 h (from 5 to 11 h), whereas in the acetate → acetate system it also takes 6 h (from 2 to 8 h). Since 70% of ascus formation is over by 16 h after the shift in Roth-Halvorson medium (33), and at least 24 h is required in glucose to acetate medium, later events must be slower in the latter medium. Evidence for this also comes from a comparison of the timing of binucleate cells, since binucleate cells in the acetate → acetate (stage III in [42]) begin to appear 4 h before DNA synthesis in the culture is complete, whereas Tingle et al. find a 1-h delay between the completion of DNA replication and stage III. Thus, a 3-h difference in the onset and termination of DNA synthesis is accompanied by a 7-h difference in the onset of meiosis I.

Our data indicate that under our conditions there is a remarkably short time between premeiotic DNA synthesis and meiosis I, considerably less than 2 h. Significantly, the kinetics of intragenic recombination seem to be virtually identical with those of DNA synthesis as originally noted by Roth and Fogel (32).

A second intent of this work was to define which of the events measurable during sporulation are specific to the sporulation process. Our results and those of others show that the processes which occur uniquely in sporulating cells are: premeiotic DNA synthesis (34, our data), meiotic recombination (12, 35, our data), formation of binucleate and tetranucleate cells, and glycogen breakdown (17, our data; Kane, Roth, and Erwin, Abstr. Annu. Meet. Amer. Soc. Microbiol., 1972). RNA and protein breakdown differ quantitatively in sporulating and nonsporulating cells and are therefore partially specific to sporulation. Biosynthesis of glycogen, RNA, and protein, loss of respiration, and rise in culture medium pH occurred similarly in both sporulating and nonsporulating cultures. Therefore, we attribute these latter events to a generalized cell response to nitrogen starvation—a response which may be necessary, but not sufficient to induce sporulation.

It was surprising to find that rates of RNA and protein synthesis are similar in sporulating and nonsporulating isogenic diploids. This finding should be useful in interpreting studies of asporogenic mutants which exhibit alterations in the gross pattern of macromolecular synthesis. From the present data it would appear that such aberrations would most likely not be specific to the developmental process per se, but would involve dislocations in the process of adjusting to starvation conditions.

Of the biochemical processes we have measured, only DNA synthesis, glycogen breakdown, and extensive RNA and protein breakdown occur uniquely in a/α sporulating cells. We wish to consider now the functional relationship between synthesis and breakdown of macromolecules and the processes of meiosis and sporulation.

In both a/α sporulating and a/a and α/α strains, the energy metabolism of yeast cells responds to nitrogen starvation, the nutritional shift which initiates sporulation in a/α diploids. Formation of glycogen, presumably via gluconeogenesis from medium acetate, may well be necessary for subsequent spore development; however, the homozygous mating type diploids synthesize as much glycogen as do a/α diploids. The pattern of overall respiratory activity versus time in sporulation medium is also the same for sporulating and nonsporulating diploids.

Taken together with our data on macromolecule synthesis, this suggests that the total energy consumption of yeast cells in this medium is not greatly affected by sporulation. Sporulating a/α diploids initiate glycogen breakdown at the time respiration has fallen to its minimal value. Whether the glucose residues are redistributed from glycogen to spore and ascus wall polysaccharides or are broken down to provide energy remains to be determined, although probably both events occur to some extent.

RNA and protein breakdown are strongly correlated with the sporulation process. At the earliest times measured, prelabeled RNA and protein are degraded, respectively, 2.7- and 2.0-fold more rapidly by sporulating than by nonsporulating diploids. This correlates very well with the finding by Chen and Miller (1) that sporulating diploids of *S. cerevisiae* exhibit a protease activity that is not present in nonsporulating cells. They did not give the reason for the failure of the latter cells to sporulate. There is evidence from protein turnover experiments (A. Hopper, unpublished data) that the total breakdown of vegetative proteins considerably exceeds the net breakdown (Fig. 5). Such macromolecule breakdown, which has been observed in nongrowing microbial and mammalian cells (2, 14, 21, 22), might occur during sporulation for one or more of the following reasons: (i) It may occur to reduce the total amount of cytoplasmic protein and RNA in preparation for the dormant ascospore state. The extensive breakdown of vegetative RNA, accompanied by excretion of the nucleotides, makes such a reduction plausible. (ii) It may occur to allow for the large qualitative changes in protein content accompanying meiosis and sporulation. Breakdown of preexisting proteins could accomplish differentiation both by removal of vegetative protein molecules and by providing amino acids for biosynthesis of new proteins. (iii) It may occur to replace old (vegetative) macromolecules with otherwise identical newly synthesized macromolecules. This seemingly wasteful process occurs in *Lillium* meiosis, where ribosomes are first degraded and later resynthesized (5). Degradation and subsequent resynthesis of the same protein or RNA molecule during meiosis can accomplish a type of cytoplasmic differentiation when two special conditions both apply: the molecules being degraded are coded for by the vegetative (diploid) genome, whereas new molecules are being synthesized by the daughter spore (haploid) nuclei; macromolecules synthesized by each daughter spore nucleus remain confined to the cytoplasm of that particular spore. In cases where the parental diploid cell is heterozygous, synthesis and degradation act to make the phenotype of each haploid cell the same as its genotype. Such a subtle cytoplasmic differentiation during meiosis has been observed for a cycloheximide-resistance marker affecting ribosome structure (A. Hopper, unpublished data).

The expectation that new, sporulation-specific proteins should be found in developing asci is based upon the results of several previous investigations. Morphogenetic events in sporulation include the formation of meiotic spindle plaques, synaptonemal complexes, and ascospore walls (B. Byers, personal communication; 25) which are essentially absent from vegetative cells. Inhibition of protein synthesis at any time prior to ascus formation blocks further development (8; A. K. Hopper and P. T. Magee, unpublished data). That some of this essential protein synthesis consists of sporulation-specific proteins is suggested by the existence of conditional mutants which are heat sensitive for sporulation, but not for vegetative growth (7). Despite these expectations, our studies on protein synthesis have shown a remarkable degree of similarity between the time course of total protein synthesis and the electrophoretic pattern of newly synthesized proteins in sporulating (a/α) and nonsporulating cells. The difference between both of these patterns and that observed in vegetative cells argues against the view that similar patterns simply reflect a low degree of resolution. The similarity in newly synthesized proteins observed in sporulating and nonsporulating cells (Fig. 4) and the constancy of the pattern observed at different times in cells undergoing meiosis suggest that proteins involved specifically in meiosis are not being observed. The true meiotic proteins may be overlooked in gel electrophoresis experiments because they are few in number and low in concentration as compared to other newly synthesized proteins. Esposito et al. (9) concluded that the number of genes which function uniquely in sporulation is small. By an analysis of the frequency of occurrence of sporulation-deficient mutants at various loci, these authors concluded that only 48 $\pm$ 27 genes function specifically in sporulation, as compared with an estimated total of 12,000 genes in the yeast nuclear genome.

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