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# Protein Synthesis in Relation to Sporulation and Meiosis in Yeast

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The dependence upon protein synthesis of physiological and biochemical events occurring during yeast sporulation was investigated. Protein synthesis was inhibited by cycloheximide. There was an early, irreversible sensitivity to inhibition with respect to cell viability and ascus formation; inhibition was reversible only if the cells were inhibited after, but not prior to, 2 to 3 h in sporulation medium. Interruption of protein synthesis of any time during sporulation inhibited all measurable metabolic and sporulation-specific processes except protein breakdown and, to some extent, ribonucleic acid synthesis. The time interval between the occurrence of an event and the protein synthesis necessary for that event was determined to be 2 to 3 h for ascus formation, $\leq$30 min for deoxyribonucleic acid synthesis, 1 h for tetranucleate cell formation, $\leq$30 min for ribonucleic acid breakdown, 1 to 2 h for glycogen synthesis, and 2 to 3 h for glycogen breakdown.

In a previous communication (7), we described the chronological order of some of the physiological events occurring during meiosis and ascospore development in yeast. Some of these happen only in cells undergoing meiosis, whereas others are apparently part of a generalized response to the starvation conditions which induce sporulation. To determine the interdependence of these processes, a means must be found to block one specifically. Then other parameters can be measured to see whether other processes depend upon the occurrence of the blocked event. Blockage can be accomplished in several ways: by use of conditional mutants in meiosis and sporulation; by use of similar mutants in some macromolecular biosynthetic process, such as protein synthesis; or by use of inhibitors which specifically block some macromolecular process.

In studying sporulation in yeast, other investigators have used inhibitors of mitochondrial development (11): the amino acid analogue ethionine (1), and cycloheximide, a ribosomal inhibitor of eukaryotic protein synthesis (4). In these experiments the major criterion for effectiveness of the inhibitor was interference with ascus formation. Except for Croes' (1) measurement of deoxyribonucleic acid (DNA) synthesis in ethionine-treated cultures, no attempt has been made to monitor any of the biochemical and physiological changes occurring during meiosis. Thus, no determination could be made of which of these events are dependent upon mitochondrial development or protein synthesis and which are not coupled to these processes. In addition, in all of the above experiments sporulation was induced by a shift of early stationary-phase, glucose-grown cells to acetate sporulation medium. This regimen requires that the cells complete respiratory adaptation in addition to undergoing the developmental program leading to meiosis and ascospore development. This complication can be avoided, as demonstrated by Roth and Halvorson (12), by shifting a logarithmic culture pregrown on acetate to sporulation medium.

Using this regimen, we investigated the dependence on protein synthesis of physiological and biochemical events occurring during yeast sporulation. We attempted to resolve these relationships by inhibiting protein synthesis and monitoring the other parameters. Protein synthesis was blocked both by cycloheximide and by the use of a mutant normal at 23 C but unable to initiate protein synthesis at 33.5 C, a temperature within the range where sporulation occurs.

## MATERIALS AND METHODS

**Yeast strains and culture conditions.** AP-1-$\alpha/\alpha$ and the culture methods used to induce sporulation were described previously (7). At 0, 2, 4, 6, and 8 h

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after the shift to sporulation medium, samples of the sporulation culture were removed and added to a prewarmed culture flask containing 100 μg of cycloheximide per ml. These subcultures and the parent culture were then sampled at hourly intervals to measure the various physiological parameters. AP-1-187 (AP-1-prt-1[18]) was constructed similarly to AP-1-$a/\alpha$. ts187, a haploid strain derived from A364A which is temperature sensitive for the initiation of protein synthesis (5), was mated with $\alpha_1$131-20. The $a/\alpha$ diploid strain was exposed to 1 min of ultraviolet irradiation, as described previously, to induce mitotic recombination leading to homozygosity at the ts187 locus. Temperature-sensitive diploid colonies were confirmed to be homozygous for the original ts187 lesion by the lack of complementation between AP-1-187 segregants and the ts187 haploid. Culture methods used to induce sporulation were as usual, except that 23 and 33.5 C were the permissive and nonpermissive temperatures, respectively. At 0, 3, and 6 h after the shift to sporulation medium, samples of AP-1-$a/\alpha$ and AP-1-187 sporulation cultures at the permissive temperature were shifted to the nonpermissive temperature. These subcultures and the parent cultures were sampled periodically to determine the various physiological parameters.

**Measurement of physiological parameters.** The methods for measuring oxygen consumption, changes in the pH of the medium, protein and ribonucleic acid (RNA) breakdown, DNA synthesis, nuclear division, intragenic recombination, and glycogen content have been described (7). Protein and RNA synthesis were measured by pulse labeling, as described, with [$^{35}$S]methionine (0.75 mCi/μmol) and [$^{3}$H]adenine (10 μCi/μmol), but 100 μg of cycloheximide per ml was added to the pulse-labeling medium for cycloheximide-treated cultures. RNA synthesis was monitored by pipetting 0.05-ml samples of the pulse-labeled cells directly onto 3-mm filter disks, which were immediately immersed in ice-cold 10% trichloroacetic acid containing cold adenine and methionine. After two successive 5% trichloroacetic acid washes, the disks were dried in alcohol-ether and ether and counted in a 2,5-diphenyloxazole and 1,4-bis[2-(5-phenyloxazolyl)]-benzine toluene scintillant. Protein synthesis was measured by heating 0.05-ml volumes of the cells at 90 C for 15 min in an excess of 10% trichloroacetic acid containing cold adenine and methionine. The precipitate remaining was collected on glass-fiber filters, washed with trichloroacetic acid and ethanol, and counted as above. The residual counts in the tritium channel after this treatment were not significantly different from the calculated 7% spillover of the [$^{35}$S]methionine counts.

## RESULTS

The intent of these experiments was to determine the effect of the inhibition of protein synthesis upon physiological and biochemical events occurring during yeast sporulation. Protein synthesis was inhibited either by the addition of cycloheximide to sporulating cultures or by a temperature shift of a sporulating culture of the conditional mutant for protein initiation (AP-1-187) to the nonpermissive temperature. Both types of inhibition gave similar results, implying that the consequences observed were due to the lack of protein synthesis per se and not to an artifact of the inhibition regimen. However, the mutant data were more difficult to interpret due to altered kinetics of the processes measured in the control culture at the high temperature.

**Effects of cycloheximide on protein and RNA synthesis.** The concentration of cycloheximide used in these experiments was sufficient to inhibit protein synthesis by 90% or more for at least the first 9 h of sporulation and was effective when added as late as 8 h after the shift to sporulation medium (Fig. 1b). Together with the fact that ascus formation was decreased by cycloheximide addition as late as 12 h, this observation argues that the cells are at least partially permeable to the antibiotic throughout the sporulation cycle. This contrasts with the behavior of metabolic precursors, such as amino acids and purine bases, whose uptake is blocked by the elevated pH of the medium

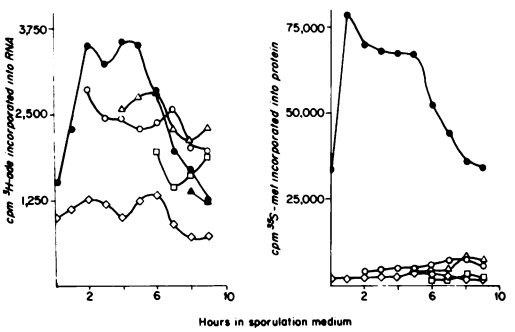


FIG. 1. *Effects of cycloheximide on protein and RNA synthesis as a function of time in sporulation medium. At 1-h intervals, two 1.5-ml volumes of cycloheximide-inhibited cells were filtered, suspended in a solution of 0.3% potassium acetate (pH 6.0) and 0.02% raffinose containing 100 μg of cycloheximide per ml, and pulse-labeled for 10 min with 50 μCi of [$^{3}$H]adenine per ml (10 μCi/μmol) and 50 μCi of [$^{35}$S]methionine per ml (0.75 mCi/μmol). Hot acid-insoluble $^{35}$S radioactivity was considered a measure of protein synthesis (b); cold minus hot insoluble $^{3}$H radioactivity was considered a measure of RNA synthesis (a). Symbols: ◇, ○, △, □, ▲, average incorporation of precursors in cultures inhibited with cycloheximide at 0, 2, 4, 6, and 8 h, respectively, after shift to sporulation medium. Control cultures (●) were the same as above, except that no cycloheximide was added to the labeling medium or to the culture.*

late in sporulation (9). The removal of cycloheximide from the cells by washing and resuspension in fresh medium led to a rapid reversal of inhibition (data not shown). Cycloheximide addition affected RNA synthesis less drastically than protein synthesis, and the nature of the effect varied according to the time of addition (Fig. 1a). When cycloheximide was added immediately after the cells were shifted to sporulation medium, RNA synthesis decreased about 30% but continued for about 9 h at a low level with a temporal pattern similar to that found in untreated cultures immediately after the shift. Addition of the antibiotic at 2 h into sporulation depressed the rate of RNA synthesis about 20%, and the rate declined slowly for the next 7 h. The decline in rate was much less rapid than in the untreated control culture, so that by 9 h the rate of synthesis in the treated culture exceeded that in the untreated one. The culture treated with cycloheximide at 4 h also showed an immediate depression of the rate of RNA synthesis to about 70% of the control rate; however, a peak in synthesis lower in absolute amount than, but otherwise similar to, the control culture appeared at 5 to 6 h. Once again, the decline in the rate was slower than in the untreated culture, and at the end of the experiment the treated cells were synthesizing RNA more rapidly than the untreated cells.

In some experiments, addition of cycloheximide at 4 h completely blocked the decline, and RNA synthesis increased for the next 5 h. We attribute this to variations in the timing of different cultures.

Inhibition of protein synthesis at 6 h depressed RNA synthesis about 30%; once again, the decline in rate was halted (in this experiment the rate actually increased after the addition of the inhibitor), and the rate at 9 h exceeded the control. At 8 h, addition of the inhibitor still caused a 25% depression of RNA synthesis.

**Viability of AP1 after cycloheximide treatment.** Since the failure of cycloheximide-inhibited cultures to sporulate might be due to its heightened toxicity on cells adjusting to starvation conditions, we tested the viability of sporulating cultures exposed to the antibiotic during sporulation. No effort was made to separate attached buds before plating, so the viability estimates are high. There appeared to be no significant separation of buds from mother cells in the course of the experiment since there was no increase in the viable counts in the control culture during the experiment. Nevertheless, Fig. 2b shows that a culture of

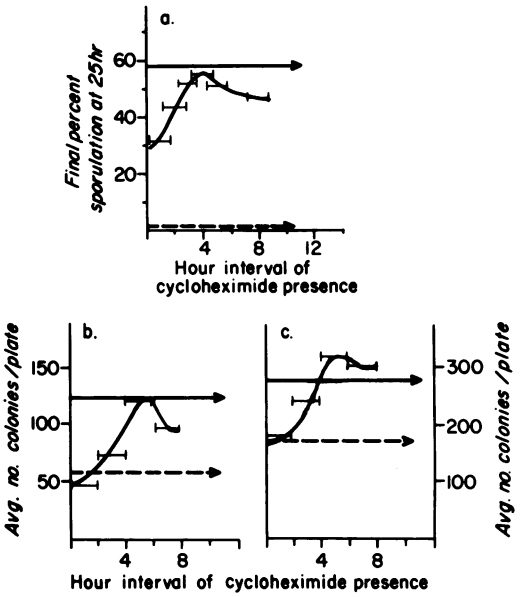


FIG. 2. *Effect of cycloheximide on sporulation and cell viability. Portions of cultures of a/α (AP-1-a/α at 1.5 × 10⁷ cells per ml) and a/a (AP-1-a/a at 2.0 × 10⁷ cells per ml) cultures in sporulation medium were treated with 100 μg of cycloheximide per ml for the hour interval indicated by the bars (——). At the termination of this interval, the cells were filtered, washed, and suspended in fresh sporulation medium. Portions of the cells were diluted to 3 × 10³ (a/α) or 4 × 10³ (a/a) cells per ml and duplicate 0.1-ml samples of each culture spread onto yeast extract peptone agar plates. The cells were allowed to grow for 2 days, at which time the average number of a/α (b) and a/a (c) colonies was determined. The remaining cycloheximide-treated a/α cells were allowed to continue sporulation until 36 h, at which time the final percentage of asci (a) was determined. Symbols: ——→, final values for an untreated control; - - - -→, final value for a continuosuly cycloheximide-inhibited culture.*

AP-1-a/α was acutely sensitive to cycloheximide from 0 to 1.5 h after it was shifted to sporulation medium. Viability was reduced some 30 to 40% by a 1.5-h exposure. Beginning at 2 h after the shift, the cells were much less sensitive to the antibiotic, and there was little loss in viability. Essentially the same results were obtained with diploids homozygous for mating type (Fig. 2c) and with AP-1-187 (data not shown) when the latter strain was subjected to temperature shifts analogous to the cycloheximide pulses outlined above. For 2.5 h after the shift to sporulation medium, sporulation ability was acutely sensitive to pulses of inhibition of protein synthesis. After this time sensitivity was not great. The data for cycloheximide inhibition are given in Fig. 2a. One obvious conclusion to be drawn

from the later part of Fig. 2a is that the effect of cycloheximide on sporulation is reversible if the antibiotic is removed.

**Effects of cycloheximide upon events unique to sporulating cells: (i) ascus formation.** Figure 3a shows the effect of cycloheximide addition, at 2-h intervals, upon the kinetics of ascus formation. Addition of the antibiotic at any time before 8 h completely blocked ascus formation. Addition at 8, 10, and 12 h greatly reduced ascus formation but did not block it completely. The rise in the percentage of asci after cycloheximide addition indicated that there is a period when ascus formation is insensitive to addition of the inhibitor. Figure 3b demonstrates that this is the case. In this figure, the final percentage of asci for each culture is plotted against the time of antibiotic addition. This curve is compared to the curve for ascus appearance in the control culture. The distance between the lines is a measure of the period before an ascus is observable, when its formation is insensitive to the inhibitor, i.e., 2 to 3 h. (Previous investigators using the glucose to acetate system have found that ascus formation is completely sensitive to cycloheximide until 14.5 h after the shift [3] and that ascus formation is reduced 30% if the inhibitor is added as late as 24.5 h after the shift.) Ascus formation in the temperature-sensitive mutant AP-1-187 was also blocked when the cells were shifted to nonpermissive temperatures at 0, 3, or 6 h (data not shown).

**(ii) DNA synthesis and nuclear division.** Inhibition of protein synthesis during mitosis in yeast prevents cells which have not entered S phase from initiating DNA synthesis (6, 15).

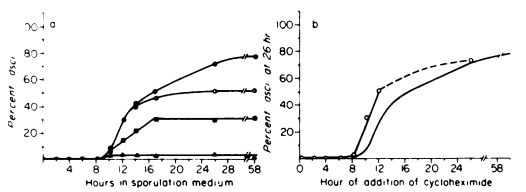


FIG. 3. *Effects of cycloheximide upon ascus formation. (a) At 2-h intervals, 0.1-ml volumes of cultures were taken and fixed with 0.9 ml of saline-formalin solution. The percentage of asci was determined by light microscopy. Symbols: ▲, ■, ◑, cultures inhibited by cycloheximide at 8, 10, and 12 h, respectively, after the shift into sporulation medium; ●, untreated control culture. No asci were detected in cultures inhibited prior to 8 h in sporulation medium. (b) Replot of data in (a). The percentage of asci at 26 h for the 8-, 10-, and 12-h inhibited cultures is presented (○) as a function of the hour of addition of cycloheximide; ——, control curve (same as ● in a).*

Figure 4a shows that premeiotic DNA synthesis was also drastically affected by such inhibition. A small amount of DNA was made in the cells treated with the antibiotic at the time of the shift to sporulation medium. Addition of cycloheximide at 2 h effectively stopped all further DNA synthesis. Addition at 4 h allowed a transient rise in DNA content; but the final level in the culture (at 24 h) was about the same as the 4-h control cell level. Addition at 6 h (data not shown) or at 8 h gave little further increase in DNA content. In both cases, there was a loss of DNA during the ensuing 20 h. Whether the slow decrease is due to lysis of cells, to instability of the newly synthesized DNA, or to some unidentified artifact complicating the assay is not clear. In AP-1-187, a shift to 33.5 C at 0, 3, or 6 h also completely blocked any increase in DNA synthesis (data not shown). The period during which DNA synthesis is insensitive to inhibition of protein synthesis was analyzed. Figure 4b shows the final DNA content as a function of the time of cycloheximide addition. Comparison to the control curve shows that, if there is a period before DNA synthesis when that process is insensitive to inhibition of protein synthesis, it is very short. Within the experimenal error in the DNA assay, the two curves are essentially indistinguishable.

Nuclear division is also very sensitive to cycloheximide. Figure 5a shows that the first nuclear division was blocked almost instantly

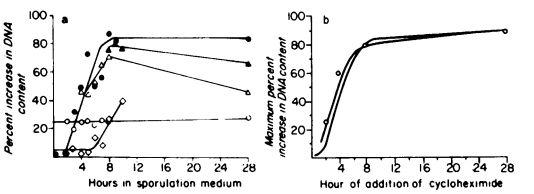


FIG. 4. *Effects of cycloheximide on DNA synthesis. (a) Cell cultures were the same as in Figure 3. At hourly intervals after the shift to sporulation medium, duplicate or triplicate 1-ml samples of each yeast culture were hydrolyzed in 1 N NaOH for 24 h at room temperature. The hydrolyzed samples were precipitated with trichloroacetic acid, washed, and dried. The resulting pellet was analyzed for DNA content by the diaminobenzoic acid fluorimetric procedure as described previously (7). Symbols: ◇, ⊙, △, ▲, average increases in DNA content of cultures inhibited by cycloheximide of 0, 2, 4, and 8 h, respectively, after the shift into sporulation medium; ●, untreated control culture. (b) Replot of data in (a). The maximal percentage of increase in DNA content for the 2-, 4-, and 8-h inhibited cultures is presented (○) as a function of the hour of addition of cycloheximide; ——, control curve (same as ● in a).*

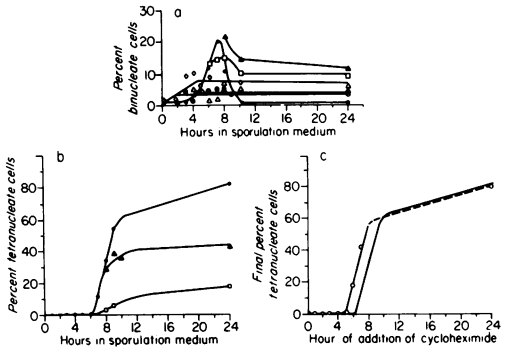


FIG. 5. *Effects of cycloheximide on nuclear division. Cell cultures were the same as in Fig. 3. At hourly intervals, 0.1-ml volumes of cells were fixed with 0.9 ml of a saline-formalin solution. The cells were collected by centrifugation, fixed to glass slides, and stained with Giemsa stain as described previously (7). (a) Percentage of cells appearing binucleate. (b) Percentage of cells appearing tetranucleate. Symbols: ◇, ⊙, △, □, ▲, subcultures inhibited with cycloheximide at 0, 2, 4, 6, and 8 h after the shift to sporulation medium; ●, an untreated control. (c) Replot of data in (b). The percentage of cells appearing tetranucleate at 24 h is presented as a function of the time of addition of cycloheximide to the cell subcultures (○): ——, control curve (same as ● in b).*

by the addition of cycloheximide at 4 h when 7% of the cells are binucleate. At 6 h, when 12% had undergone the first division, inhibition of protein synthesis allowed a small increase in binucleate cells, followed by a decline. The cultures to which cycloheximide was added early showed a few binucleate cells, but these occurred earlier than in the control culture and are, we think, artifacts due either to the toxic effects of the antibiotic or to cells blocked part way through the final mitotic division.

The second nuclear division, which occurred at about 7 h in the control cultures, was responsible for the decrease in binucleate cells and an increase in tetranucleate cells. Figures 5a and 5b show that this second division is also blocked by the antibiotic. Figure 5c indicates that the process becomes insensitive to cycloheximide about 1 h before the second division. Thus, both kinds of nuclear events, DNA synthesis and meiotic division, are blocked by cycloheximide.

**(iii) Intragenic recombination.** Since AP-1-*a*/*α* is an adenine auxotroph carrying noncomplementing mutations at the *ade2* locus, one can measure intragenic recombination during meiosis and sporulation by determining the number of adenine prototrophs in the cultures (7). Figure 6 shows the effect of cycloheximide on this process. The number of recombinants was not corrected for viability. The slight decrease in viability observed (never more than 25%, data not shown) did not alter the results. Two facts are apparent: the antibiotic very drastically arrests the process and the number of recombinants found in the treated cultures is less than were present in the parent culture at the time the shift to cycloheximide was made, e.g., control at 4 h, 150 recombinants; cycloheximide added at 4 h, 75 recombinants; control at 6 h, 220 recombinants; and cycloheximide added at 6 h, 80 recombinants. Recombination seems to be decreased when protein synthesis is inhibited.

**(iv) RNA and protein degradation.** The breakdown of protein and RNA, as measured by release of radioactive breakdown products from prelabeled cells, was shown to be much more extensive in sporulating than in nonsporulating cells (7). Figures 7a and 7b show that, whereas the rate of protein breakdown was not affected significantly by inhibition of protein synthesis, the rate of RNA degradation was quite sensitive. Except for the culture inhibited at the time of the shift to sporulation medium, the extent of breakdown is directly proportional to the time spent in sporulation medium in the absence of

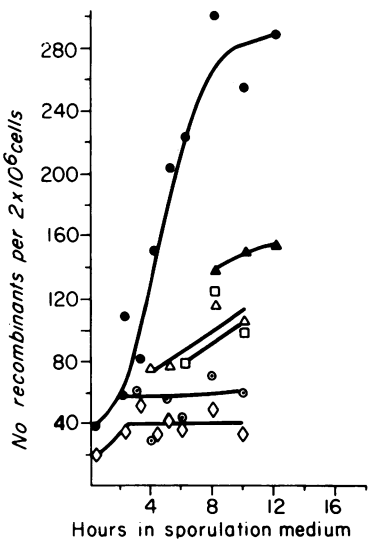


FIG. 6. *Effects of cycloheximide on intragenic recombination. Cultures were the same as in Fig. 3. At hourly intervals, two 0.1-ml volumes of the culture (equal to 2 × 10⁶ cells) were spread onto two nutrient plates lacking adenine. The resulting average number of colonies per plate was taken as the number of intragenic recombinants per 2 × 10⁶ cells. Symbols: ◇, ⊙, △, □, ▲, cell subcultures inhibited by cycloheximide at 0, 2, 4, 6, and 8 h into sporulation; ●, untreated control culture.*

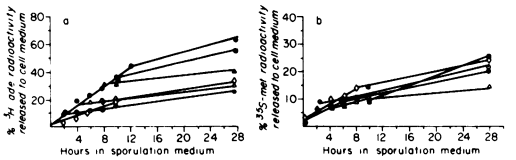


FIG. 7. *Effects of cycloheximide upon RNA and protein degradation. Cell cultures were grown as described previously. Six generations prior to sporulation, 2 μCi of [$^3H$]adenine per ml (3.0 Ci/mmol) and 2.75 μCi of ml [$^{35}S$]methionine per ml (34 Ci/mmol) were added to the vegetative medium. When the cultures had reached a density of 2.0 × $10^7$ cells per ml, the cultures were filtered, washed, and resuspended in sporulation medium. The amount of radioactive precursor in high-molecular-weight RNA and protein was determined. At 2-h intervals, two 1-ml volumes of cells were centrifuged, 20 μliters of the cell medium was spotted onto Schleicher & Schuell 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. (a) [$^3H$]adenine or RNA broken down and released. (b) Percentage of [$^{35}S$]methionine or protein broken down and released. Symbols: ◇, ⊙, △, ▲, ■, cell subculture inhibited by cycloheximide at 0, 2, 4, 8, and 10 h, respectively, after the initiation of sporulation; ●, the untreated control culture.*

the inhibitor. Addition of cycloheximide at any time during sporulation appears to reduce abruptly the rate of breakdown. The rate of RNA and protein breakdown was enhanced by shifting AP-1-*a*/α cultures from 23 to 33.5 C. For protein breakdown, this enhancement was the same in the conditional mutant (AP-1-187) for protein initiation as in AP-1-*a*/α strain. No increase in the rate of RNA breakdown occurred in AP-1-187 if the temperature was raised, although RNA breakdown was not arrested by such a shift.

**(v) Glycogen synthesis and breakdown.** Figure 8a shows the glycogen content of the various cultures as a function of time in sporulation medium. Both the synthesis and the breakdown of glycogen are dependent upon protein synthesis. Cycloheximide addition at 2 or 4 h stopped further accumulation of the carbohydrate, whereas the culture treated at 6 h accumulated an excess, apparently due to the failure to synthesize the enzyme needed for breakdown. Addition at 8, 10, or 12 h led to a fairly rapid stabilization of the glycogen content. Figure 8b shows the glycogen data plotted similarly to Figure 2b. In this case, the period of insensitivity appears to be 1 to 1.33 h long for synthesis and slightly longer, perhaps 3 h, for degradation.

## DISCUSSION

In designing these experiments, we attempted to determine which events occurring during meiosis and sporulation in yeast are dependent upon protein synthesis, and when the necessary proteins are synthesized. All the processes described previously (7), except protein breakdown and, to some extent, RNA synthesis, require protein synthesis. We cannot, of course, distinguish between an event which itself requires a new gene product and one in a developmental program whose initiation is dependent upon protein synthesis.

We analyzed the time at which a particular process becomes insensitive to the inhibition of protein synthesis. The period of insensitivity was determined by the distance between the curve derived by plotting the final value obtained for each inhibited culture against the time of antibiotic addition and the curve derived by plotting the value of an event in an uninhibited control culture against the time the event was measured (Fig. 3b). For example, sensitivity to cycloheximide ceased for ascus formation at 2 to 3 h before asci were observable; DNA synthesis, on the other hand, was sensitive up to 30 min or less before the process began. It is tempting to argue that the beginning of such a period of insensitivity marks the

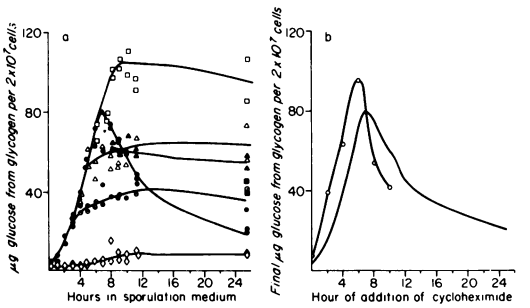


FIG. 8. *Effects of cycloheximide on glycogen synthesis and degradation. Cultures were the same as described in Fig. 3. (a) At hourly intervals, two 5-ml volumes of each culture were centrifuged and frozen. Glycogen was precipitated from the thawed cultures and enzymatically broken down to glucose, and the resulting micrograms of glucose per ml of cells was determined by the glucose oxidase method as described previously (7). Symbols: ◇, ⊙, △, □, ▲, ■, cell subcultures inhibited by cycloheximide at 0, 2, 4, 6, 8, and 10 h, respectively, after the initiation of sporulation; ●, untreated control culture. (b) Replot of (a). The glucose from glycogen per 2 × $10^7$ cells at 25.5 h is presented as a function of time of inhibition by cycloheximide (○); ——, untreated control (same as ● in a).*

time at which the proteins involved in any particular event are synthesized. However, such an interpretation is complicated by the fact that protein synthesis is inhibited only 75 to 95% by the antibiotic (Fig. 1). The insensitive period may, therefore, indicate only the time at which the last required gene product sensitive to cycloheximide is synthesized.

There was a rapid loss in viability when cells were shifted to an anitrogenous acetate-containing medium, and protein synthesis was inhibited. If protein synthesis was arrested within 1 to 1.5 h after the shift to sporulation medium, the cells, whether or not they are able to sporulate, suffered a drastic loss in viability. This was accompanied by a failure to incorporate protein and RNA precursors, by a rapid decline in $O_2$ consumption (data not shown) (in contrast to the transient rise seen in uninhibited cells), and by a failure to begin either sporulation-specific processes or the nonspecific events associated with starvation. We assume that we have affected some fundamental adaptation to sporulation medium which requires protein synthesis, but we have no idea what the process is.

Ascus formation was inhibited by the addition of cycloheximide at any time up to 2 h before the structure became visible in a light microscope. The sensitivity of this process is not surprising, since as the final product of the developmental program the ascus formation must be dependent upon successful completion of a large number of events. Furthermore, it seems quite likely that a number of unique proteins must be present in the spores; the 2-h period of insensitivity to cycloheximide may indicate that all the necessary precursors are present at that time.

RNA synthesis was not drastically inhibited by cycloheximide, and in fact continued at a significant rate for several hours after the addition of the antibiotic (except for the culture inhibited at the time of the shift to sporulation medium). The latter part of the peak of RNA synthesis, at 5 to 7 h, was eliminated if protein synthesis was arrested at 2 h, but it seemed to appear, although decreased in magnitude, if the antibiotic was added at 4 h. One explanation for this would be the requirement for a new transcription factor synthesized between 2 and 4 h. The most striking feature of RNA synthesis in the inhibited cultures was the late rise in rate, at a time of decreasing rate in the control culture. This behavior would be expected if turnoff of RNA synthesis were controlled positively by a short-lived system requiring protein synthesis.

Roth and Dampier (13) have demonstrated that addition of cycloheximide to mitotic yeast is followed within 15 min by a decrease in the rate of RNA synthesis as measured by [$^3$H]uracil incorporation. Our results are in striking contrast to these. Although part of the difference may be due to experimental differences (our strain is a diploid heterozygous for a cycloheximide-resistance locus; we used adenine rather than uracil as a label), we observed the same phenomena in measurements of protein and RNA synthesis in AP-1-187 at the nonpermissive temperature (data not shown). Therefore, there appears to be a fundamental difference in the control of RNA synthesis during meiosis as compared to mitosis. The cells now appear to be "relaxed," at least vis a vis cycloheximide. If very different kinds of RNA are being made during ascosporogenesis (for example, much less ribosomal RNA than during vegetative growth), we may be seeing here a mode of regulation that is masked under normal culture conditions.

Glycogen synthesis and degradation appear to be dependent upon the synthesis of new proteins. Since accumulation of this storage compound is a response of sporulating or nonsporulating yeast to starvation for nitrogen, the enzymes are probably derepressed by the shift, but relatively asynchronously in the culture. Thus, the relatively rapid cessation of synthesis after addition of cycloheximide at 2 and 4 h probably reflects the proportion of the culture which has completed synthesis of the enzymes and has accumulated its complement of glycogen, not a requirement for continued protein synthesis to make glycogen. The same explanation may hold for the "overshoot" in the culture inhibited at 6 h; the peak in the control is the sum of synthesis and breakdown. Alternatively, the switch from accumulation to degradation may be controlled by a protein whose synthesis is blocked by cycloheximide. Since glycogen degradation is one of the events unique to sporulating cells, the enzymes (or regulatory proteins) responsible for it must be classed among the gene products unique to sporulation. We can say from these data that at least one of the essential gene products is synthesized between 6 and 10 h.

Croes found premeiotic DNA synthesis to be sensitive to the addition of an amino acid analogue. We found a similar sensitivity to cycloheximide. Protein synthesis appeared to be required at least up to 15 min before initiation. In mitotic DNA synthesis in yeast, Hereford and Hartwell (6) and Williamson (15) have

found that initiation, but not continuation, of DNA replication is inhibited by cycloheximide. If we assume that the same is true during meiosis, the apparent arrest of synthesis part way through the cycle may be interpreted as due to the asynchrony of the culture. Alternatively, DNA synthesis may be controlled very differently during meiosis as compared to mitosis. The reason for the decline in DNA content during the last 8 h of the experiment is not readily apparent. We have not detected cell lysis brought about by prolonged incubation in the presence of cycloheximide in these cultures, but it is possible that some occurs.

Completion of premeiotic DNA synthesis is not sufficient for the second nuclear division. Addition of the inhibitor at 6 h, which allows the final level of DNA synthesis to reach 75% that of a normal culture, drastically inhibited (85%) the second nuclear division. Accompanying this inhibition was an apparent decrease in the number of binucleate cells. This decline may be analogous to the decrease noted in some conditionally asporogenous mutants cultivated at the restrictive temperature. In the latter case, it has been attributed to recondensation of the chromatin (P. Moens, personal communication). The dependence of nuclear division on protein synthesis is not really surprising since the spindle apparatus, largely protein, must be elaborated before nuclear division can occur. Our data indicate that some essential components must be made as late as 30 min before division.

The drastic effect of inhibition of protein synthesis on RNA degradation contrasts with the lack of effect on protein degradation. The immediate change in the rate of release of acid-soluble RNA fragments could be explained if the antibiotic were interrupting the synthesis of an enzyme which continues to be elaborated throughout the sporulation cycle. However, one might then expect that the residual rate would be higher the later the inhibitor were added, which does not seem to be the case. Such an enzyme would have to have a relatively short half-life to explain the present data. An alternative explanation is that only free ribosomal subunits are available for degradation, and cycloheximide, by stabilizing polysomes, inhibits the appearance of subunits and prevents this. The residual rate of degradation would then correspond to breakdown of transfer and messenger RNA. The data for AP-1-187 RNA breakdown do not confirm this possible explanation, since polysomes break down in this mutant under mitotic growth conditions at the restrictive temperature.

The control culture showed a rapid decline in the rate of RNA synthesis after 6 h; the inhibited cultures showed a much more gradual decline. If the decrease in RNA degradation decreases the pools of the inhibited cultures, the added isotope would have a higher effective specific activity, so the apparent rate of synthesis would be higher. Although the pools do not fluctuate drastically in normally sporulating cultures, we did not measure them in inhibited cultures.

The most surprising effect of the inhibitor was its "decrease" of recombination. This may mean the recombinants are more sensitive than the bulk of the population to inhibition of protein synthesis; the decrease in recombinants would then be due to their loss of viability. Death would have to be quite rapid under the circumstances, however, since the inhibited cultures were diluted and plated for the first point in the measurement of recombination within 20 min after addition of the antibiotic and, as mentioned above, the inhibition of protein synthesis is easily reversible. The recombination process itself may be quite sensitive to inhibition of protein synthesis, and a partially completed recombination event may be reversible if protein synthesis is arrested. In meiotic microsporocytes of *Lillium*, addition of cycloheximide in late zygotene leads to disappearance of the synaptonemal complex (10). Although structures strictly analogous to synaptonemal complexes have not been observed in yeast, it is interesting to speculate that an analogous effect in yeast might very well affect intragenic recombination.

It is illuminating to compare our results with *Saccharomyces cerevisiae* undergoing meiosis with the findings for plants of the genus *Lillium*. These cells progress from premeiotic S phase through meiosis under proper conditions. During this process, *Lillium* cells degrade 95% of their old ribosomes (2), whereas yeasts degrade up to 70% of their RNA during sporulation. Yeasts require protein synthesis at least for the initiation of premeiotic DNA synthesis; so does *Lillium* (10). Since meiosis in these organisms is more extensively studied, such comparisons should become very helpful in exploring the universal aspects of meiosis.

A few general conclusions can be drawn from the experiments about the developmental program leading to meiosis and ascospore development in yeast. The first is that all events which we measured, except protein degradation, are dependent upon protein synthesis for their initi-

ation, although the period of the requirement varies with the event. Only RNA breakdown appears to be continuously dependent upon protein synthesis. We have defined a new period of adaption to sporulation medium requiring protein synthesis.

Our lack of success in differentiating among the various developmental subprograms by the use of cycloheximide is most likely due to lack of sensitivity of the tool rather than to the lack of existence of these programs. More fruitful approaches may be made by the use of conditional meiotic mutants of the sort which have been isolated and partially characterized (3). It is important that the processes which have been shown to be unique to sporulation, such as RNA and glycogen breakdown, be carefully studied under nonpermissive conditions in these mutants. Such studies may unravel some of the complex relationships among the events in ascospore development.

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