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Acetate Utilization and Macromolecular Synthesis During Sporulation of Yeast

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Acetate utilization and macromolecule synthesis during sporulation (meiosis) of *Saccharomyces cerevisiae* were studied. When diploid cells are transferred from glucose nutrient medium to acetate sporulation medium at early stationary phase, respiration of the exogenously supplied acetate proceeds without any apparent lag. At the completion of ascospore development, 62% of the acetate carbon consumed has been respired, 22% remains in the soluble pool, and 16% is incorporated into lipids, protein, nucleic acids, and other cell components. Measurements of the rate of protein synthesis during sporulation reveal two periods of maximal synthetic activity: an early phase coincidental with increases in deoxyribonucleic acid, ribonucleic acid, and protein cellular content and a later phase during ascospore formation. Experiments in which protein synthesis was inhibited at intervals during sporulation indicate that protein synthesis is required both for the initiation and completion of ascus development.

Under appropriate conditions, sporulation can be induced in nearly all of the members of a diploid yeast population. The process is complex and involves meiosis and subsequent ascospore formation. At present, there is little biochemical information on the differences between meiosis and mitosis and the regulatory events which determine whether a diploid cell will initiate one or the other of these processes. The recent demonstration of numerous recessive mutations in *S. cerevisiae* (8*a*; M. S. Esposito and R. E. Esposito, Genetics, vol. 60, Abstr., p. 176, 1968) and in the fission yeast *S. pombe* (2), which prevent meiosis and sporulation but which permit mitotic division, suggests that these two modes of nuclear division are under separate genetic control and hence may differ biochemically in a number of respects.

The present paper summarizes experiments designed to define the physiological stages of sporulation of homothallic strains of *S. cerevisiae* which permit isolation of meiotic conditional mutants (8*a*). To characterize the physiological changes associated with sporulation, the course of macromolecular synthesis and acetate utilization during sporulation were investigated.

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MATERIALS AND METHODS

Yeast strains. The following homothallic diploid strains of *Saccharomyces* were employed in this study:

$$\text{S41}\ \frac{a}{\alpha}\ \frac{D}{D}\ \frac{arg\text{-}4}{arg\text{-}4}\ \frac{acr\text{-}1}{acr\text{-}1}$$

$$\text{z140-9A}\ \frac{a}{\alpha}\ \frac{D}{D}\ \frac{lys\text{-}2}{lys\text{-}2}$$

The *D* gene confers a homothallic phenotype by virtue of its action during the mitotic divisions following ascospore germination. It causes directed mutations of either the *a* or α mating type allele to its opposite form; mating ensues and the spore colony produced consists primarily of diploid cells (D. C. Hawthorne, Proc. 11th Intern. Congr. Genetics, Vol. 1, Abstr., p. 34, 1963). Symbols are as follows: *a*, α, mating type alleles; *D*, diploidization gene (16); *arg*, arginine auxotroph; *acr*, actidione (cycloheximide) resistance; *lys*, lysine auxotroph.

Media. The amounts of the various ingredients are those required for the preparation of 1 liter of medium. Yeast extract peptone (YEP) consisted of 20 g of dextrose, 20 g of Bacto-peptone, and 10 g of yeast extract. Sporulation medium I consisted of 20 g of potassium acetate and 1 g of yeast extract. Sporulation medium II consisted of 20 g of potassium acetate and 75 mg of L-arginine hydrochloride. Sporulation

medium III consisted of 20 g of potassium acetate and 75 mg of L-lysine hydrochloride. To solidify any of these media, 15 g of agar was added.

Sporulation and counting procedure. Diploids to be sporulated were inoculated into liquid YEP medium at an initial cell density of 5×10^4 cells/ml and incubated with shaking at 30 C. The cells for this initial inoculum were obtained from 2-day-old colonies grown on solid YEP medium. The cells were harvested by filtration after 24 to 26 hr of growth in YEP (early stationary phase), washed twice with sterile distilled water, and suspended in sporulation medium at ca. 5×10^7 cells/ml. Tetracycline (20 mg/liter) was routinely added to sporulating cultures to retard bacterial contamination. The kinetics of the appearance of asci in these cultures was determined by hemacytometer counts. For these estimates, no attempt was made to distinguish buds from cells; each was counted as a cell. This procedure was adopted because it eliminates subjective judgments regarding the total cell number which must be made when budded cells are classified as one or two cells, depending upon the size of the bud in relation to the mother cell. The sporulation frequencies obtained by this counting procedure are minimal estimates of the percentage of cells which have sporulated, since not all buds contain nuclei. Sporulating cultures which could not be counted immediately were fixed in 4% Formalin and refrigerated. Increases in cell number due to vegetative division did not occur in the sporulation media employed.

Measurement of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein. DNA was assayed colorimetrically with the diphenylamine reagent (3); DNA from Mann Chemical Co. served as a standard. DNA was extracted from samples of 4×10^8 to 10×10^8 whole cells by the method of Ogur and Rosen (12). Nucleic acid was extracted from each sample at least twice to insure total recovery of DNA. RNA was assayed by the orcinol method described by Ceriotti (4); yeast RNA was used as a standard. Colorimetric determinations of protein content were performed with the Folin phenol reagent as described by Lowry et al. (10), with bovine serum albumen used as a standard.

Isotope incorporation. Acetate uptake by sporulating cells was determined by using ^{14}C-acetate. For each sample, approximately 4×10^8 cells which had been exposed to labeled sporulation medium were washed twice and resuspended in 2 ml of water. A sample of this cell suspension was then introduced directly into scintillation fluid for counting. From these samples, the soluble pool, lipids, nucleic acids, and proteins were extracted. The soluble pool was extracted by boiling the 2-ml water suspensions for 10 min. The samples were centrifuged, and a portion of the supernatant fluid was removed for counting. Lipids were extracted from the pellet by heating in 2 ml of ethyl alcohol-ether (3:1) for 3 min at 60 C. Two ethyl alcohol-ether extractions were performed, and the supernatant fluids were combined and counted. Nucleic acids were extracted by 10 min of boiling in 2 ml of 5% trichloroacetic acid; a sample of the supernatant fluid obtained from this extraction was also counted. Proteins were extracted from the remaining material by overnight incubation in 2 ml of 0.1 M NaOH at 37 C. A portion of each of these final supernatant fluids was counted to measure incorporation of acetate carbon into protein. The residue of cellular debris was then collected on membrane filters (0.45 μm pore size; Millipore Corp., Bedford, Mass.), dried overnight at 65 C, and suspended in scintillation fluid for counting. CO_2 evolution resulting from acetate utilization was measured by trapping the CO_2 evolved by sporulating cells in 0.3 M NaOH solutions; a sample was then introduced into scintillation fluid for counting.

Measurements of the incorporation of ^{3}H or ^{14}C or ^{14}C-amino acids as an index of protein synthesis were done as described by Rodenberg et al. (13).

Preparation of unbudded cell fractions. To obtain a single cell fraction, approximately 1.5×10^9 cells, obtained from early stationary-phase growth in YEP medium, were washed twice in sterile distilled water and suspended in 3 ml of 12% sucrose. This suspension was layered on the surface of a 40-ml, 12 to 40% linear sucrose gradient which was centrifuged at room temperature for 4 min at $1,600 \times g$. The top 20 ml of the gradient was discarded, and the next 10 ml was collected and washed free of sucrose in sterile distilled water. Whereas unfractionated cell populations routinely have approximately 37% single unbudded cells, the fractions so prepared contain greater than 80% single unbudded cells.

Dry weight measurements. The dry weight of cells was determined by collecting a known number of cells on tared filters (Millipore). The filters were dried in a vacuum oven at 85 C for 48 hr and weighed.

Materials. The chemicals were obtained from the following suppliers: acetate-*1*-^{14}*C* (13.7 mc/mmole), acetate-*2*-^{14}*C* (34 mc/mmole), ^{3}H-L-lysine (252 mc/mmole), ^{14}C-L-lysine (175 mc/mmole), and ^{14}C-L-phenylalanine (355 mc/mmole) from Schwarz BioResearch Inc., Orangebury, N.Y.; actidione from The Upjohn Co., Kalamazoo, Mich.; and tetracycline from K & K Laboratories, Jamaica, N.Y.

RESULTS

Pregrowth and sporulation. To determine the period during vegetative growth when cells transferred to sporulation medium produce asci rapidly and to a maximal level, cells were removed at intervals from a culture of diploid S41 growing in YEP medium and were transferred to sporulation medium. The growth curve of S41 in YEP medium is shown in Fig. 1. The percentage of asci present after 24, 48, and 72 hr of incubation is summarized in Table 1. Cells transferred to sporulation medium during early log phase (sample 1) did not yield asci by 24 hr, though asci eventually appeared. As early stationary phase is approached (samples 2 through 5), the rapidity with which asci are formed and the final percentage of asci observed reached maximal levels.

Biochemical changes during sporulation. Changes in the DNA, RNA, and protein content

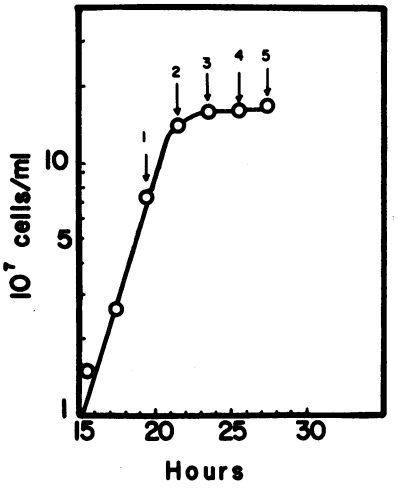

FIG. 1. *Growth of diploid S41 in YEP medium. Strain S41 was grown aerobically in YEP medium from an initial inoculum of 5 × 10⁴ cells/ml. At the time indicated by the arrows, cells were transferred to sporulation medium I.*

TABLE 1. *Effect of physiological age on sporulation*[a]

Sample no.	Hr in growth medium	Cells/ml in growth medium	Percentage of asci at[b]		
			24 hr	48 hr	72 hr
1	19.5	7.2×10^7	0	12	63
2	21.5	1.4×10^8	38	59	68
3	23.5	1.6×10^8	51	69	75
4	25.5	1.7×10^8	52	70	78
5	27.5	1.7×10^8	52	70	78

[a] See Fig. 1 for collection of samples.

[b] Percentage of sporulation at 24, 48, and 72 hr in sporulation medium of diploid S41 withdrawn from growth medium at time indicated.

of cells undergoing sporulation are summarized in Fig. 2A. DNA synthesis occurred from T_4 to T_{12} and achieved a percentage increase equivalent to the total ascus production (Fig. 2B). The RNA and protein content per cell increased to maximal levels at T_{10} and subsequently declined. By 50 hr, the protein cell content returned to its initial value, whereas the RNA cell content remained approximately 20% higher than the value observed at T_0. During sporulation, the dry weight of cells increased 60% over the initial value by T_{12} and slowly declined thereafter (Fig. 2B). Before the appearance of asci, the *p*H of the supernatant medium increased from 7.0 to 8.5.

Acetate metabolism. At the period of growth in nutrient medium, when transfer of diploid S41 to sporulation medium results in rapid ascus formation, the population consisted of approximately 37% single unbudded cells and 67% budded cells and higher multiples. It was of interest to inquire whether a single cell fraction prepared from the total population would demonstrate the same rate of acetate consumption and ascospore development as an unfractionated population. A single cell fraction obtained by sucrose density centrifugation, as previously described, and an unfractionated cell population were compared with respect to the rate of CO_2 evolution upon transfer to sporulation medium containing acetate-*1*-^{14}C (Fig. 3). The single cell fraction and the unfractionated cells sporulated at the same rate and to the same level (65%). Evolution of CO_2 from acetate oxidation began without any apparent lag (Fig. 3 insert). The initial rate of CO_2 evolution of both populations was identical, indicating that a single cell fraction is not different from the overall population with respect to ability to respire acetate upon transfer to sporulation medium. With time (after T_{25}),

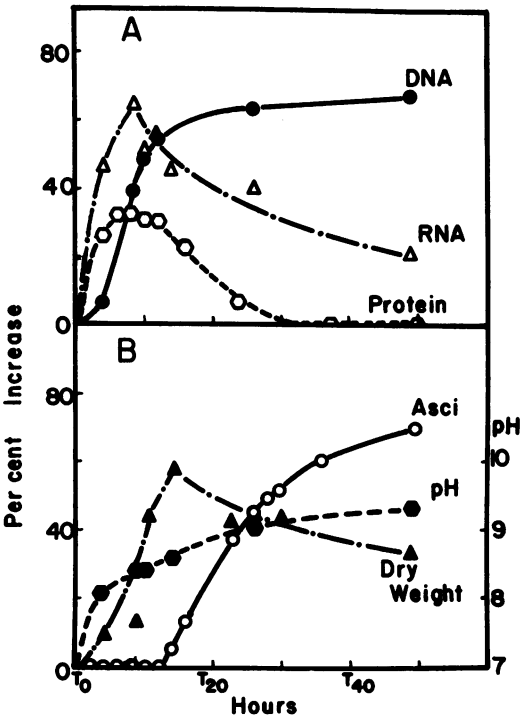

FIG. 2. *Biochemical changes during sporulation of strain S41 in sporulation medium II. Macromolecular synthesis (A); ascus production, dry weight, and pH increase (B). Values for macromolecules are given as per cent increase above T_0 values: DNA = 3.02 μg/10⁸ cells, RNA = 371 μg/10⁸ cells, and protein = 658 μg/10⁸ cells.*

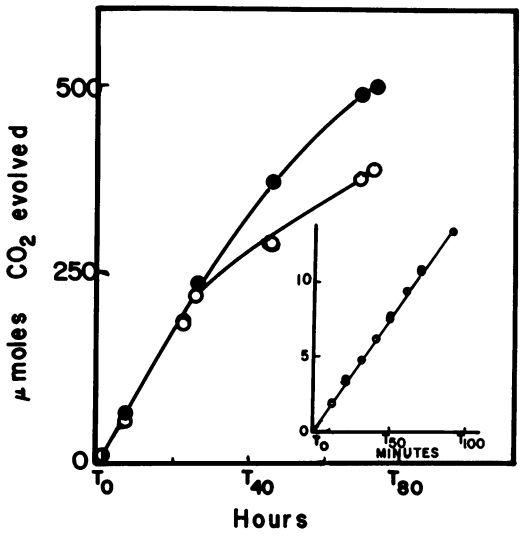

FIG. 3. *Evolution of CO_2 during sporulation of strain S41. Abscissa is micromoles of CO_2 evolved per 6.4 × 10^8 cells in sporulation medium II containing acetate-1-^{14}C (1.33 μc per 20 mg per ml); (○) single cells, (●) unfractionated cells. Insert: short-term evolution of CO_2. (The rate of ascus formation of the unfractionated cells and of the single cell fraction was identical to that shown for diploid S41, Fig. 2B.)*

however, the rate at which acetate is respired by the single cell fraction decreased, so that by T_{72} only 380 μmoles of acetate-derived CO_2 evolved in comparison to 500 μmoles by the unfractionated cells.

The utilization of acetate during sporulation has been studied by utilizing acetate-2-^{14}C. For these experiments, cells from an early stationary-phase population of diploid S41 were introduced into sporulation medium containing the isotope and the evolution of ^{14}C-CO_2, and the incorporation of acetate by cells was followed (Fig. 4). The evolution of CO_2 proceeds linearly through the completion of ascus development, whereas the total incorporation of acetate by cells achieves a maximal level by T_{24}. The distribution of the acetate incorporated by cells (Fig. 4) into the soluble pool, lipids, proteins, nucleic acids, and residue of cellular debris is shown in Fig. 5. The kinetics of incorporation of acetate-2-^{14}C carbon into protein and nucleic acids closely follow the bulk increases in cell content of these macromolecules. The incorporation of acetate into lipids proceeds in two stages. The first stage is completed by T_{10}, while the second phase occurs from T_{22} to T_{30}. Exclusive of the soluble pool, the incorporation of acetate-derived carbon into the residue fraction is proportionately greater than into other cell components.

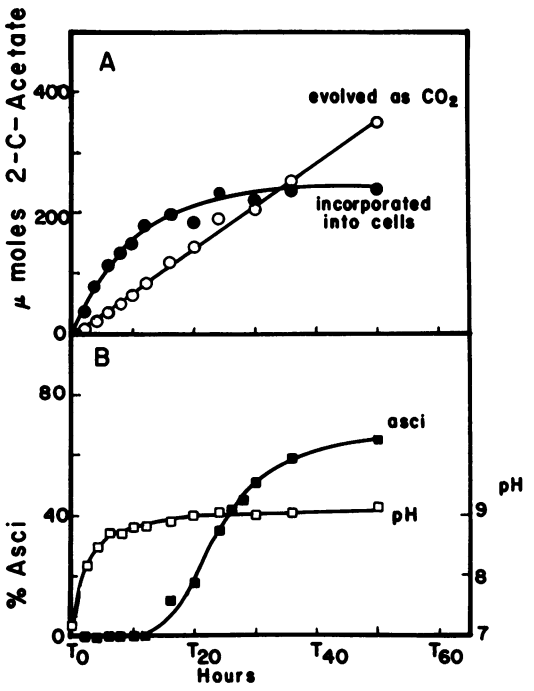

FIG. 4. *Utilization of acetate by strain S41 during sporulation in medium containing acetate-2-^{14}C. Specific activity was 1.33 μc per 20 mg per ml. (A) Micromoles of CO_2 evolved and micromoles of acetate-2-^{14}C incorporated into cells per 6.4 × 10^8 cells. (B) Ascus production and pH increase during sporulation.*

Protein turnover and protein synthesis during sporulation. Cells of diploid Z140-9A were grown in YEP containing ^{3}H-L-lysine and then transferred to unlabeled sporulation medium. After an initial incorporation of ^{3}H-L-lysine (from the soluble pool) into proteins, the ratio of ^{3}H-lysine counts per minute at Tx/To (Fig. 6) steadily declined from T_{10} to T_{30} and then reached a plateau (Fig. 6A). This result is compatible with the net changes in protein content of cells during sporulation (Fig. 2A).

To determine whether the rate of protein synthesis varies during sporulation, sporulating cells were pulse-labeled with ^{14}C-L-lysine throughout the sporulation cycle. At intervals, a sample of sporulating cells was withdrawn from a single sporulating culture, isotope was added, and samples were removed for counting at 0, 1.5, and 3 hr after addition of isotope. The results of this experiment are summarized in Fig. 6B. Two periods of maximal protein synthesis were observed at T_4 to T_7 and T_{23} to T_{27}. The latter peak period of protein synthesis is coincidental with the second phase of acetate-2-^{14}C incorporation into lipids (Fig. 5).

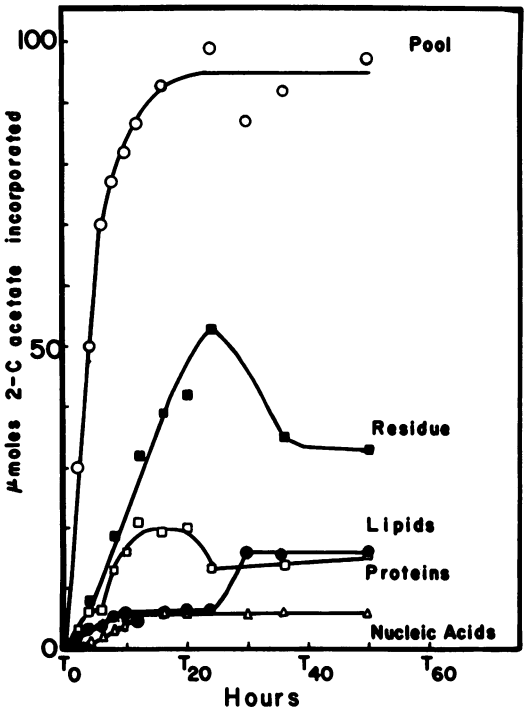

FIG. 5. *Incorporation of acetate-2-^{14}C into the soluble pool, lipid fraction, nucleic acid, protein, and residue extracts during sporulation of strain S41. Medium: sporulation medium II containing acetate-2-^{14}C (1.33 μc per 20 mg per ml).*

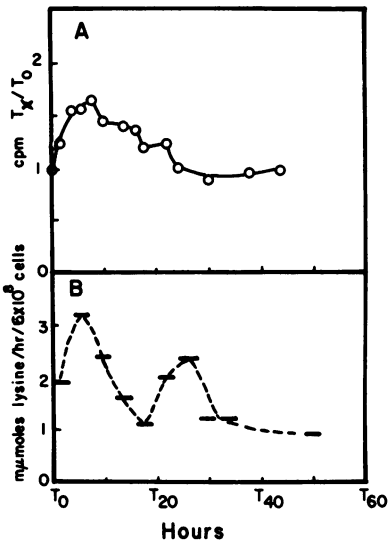

FIG. 6. *Protein synthesis and turnover during sporulation of strain Z140-9A. (A) Protein turnover. The culture was grown in YEP medium containing 2.5 μc of ^{3}H-L-lysine/ml. When the culture reached the early stationary phase, the culture was harvested, washed, and suspended in sporulation medium III. The trichloroacetic acid-precipitable counts were measured initially (T_0) and at intervals (T_x) after the culture was transferred to sporulation medium. The ratio of T_x/T_0 is plotted as a function of time during sporulation. (B) Rate of protein synthesis. At intervals during sporulation in medium III, lysine-^{14}C-L (0.9 μc/ml) was added and the rate of isotope incorporation was followed into hot trichloroacetic acid-precipitable material over a 3-hr period. The bars refer to the average rate of lysine incorporation over the 3-hr period for each sample.*

Inhibition of protein synthesis during sporulation. As shown in Fig. 6, although protein synthesis occurs throughout sporulation, its rate varies considerably. To determine whether continuous protein synthesis is required for sporulation, actidione (cycloheximide), an inhibitor of protein synthesis, was added to sporulating cultures and its effect on ascospore production was observed.

When actidione is added to cells (sensitive to this inhibitor) in sporulation medium, the onset of protein synthesis as measured by the incorporation of ^{14}C-L-lysine into protein is prevented (Table 2). To ascertain the effect of inhibiting protein synthesis at various times during sporulation, a series of sporulating cultures of diploid Z140-9A, prepared from the same starting inoculum, were exposed to actidione at several different times after introduction into sporulation medium. The results of this experiment are summarized in Fig. 7. When protein synthesis is inhibited before $T_{14.5}$, no asci are formed. When actidione is added after asci have begun to appear, ascus formation proceeds for a short time and then ceases. The total percentage of asci present at

TABLE 2. *Effect of actidione on protein synthesis during sporulation*

Hr in sporulation medium	Percentage of asci		Incorporation of lysine[a]	
	−Actidione	+Actidione[b]	−Actidione	+Actidione[b]
0	0	0	0	0
4	0	0	68.7	0
8	0	0	85.2	0
16	0.8	0	96.6	0
20	36.3	0	98.6	0.4
24	49.7	0	96.0	0.4
32	70.0	0	95.5	2.8
48	71.0	0	94.1	11.0

[a] Nanomoles of lysine incorporated into trichloroacetic acid-precipitable material per 7.8 × 10^8 cells.

[b] Cells of Z140-9A were placed in sporulation medium III containing ^{14}C-L-lysine (0.9 μc/ml) and, where indicated, actidione (50 μg/ml).

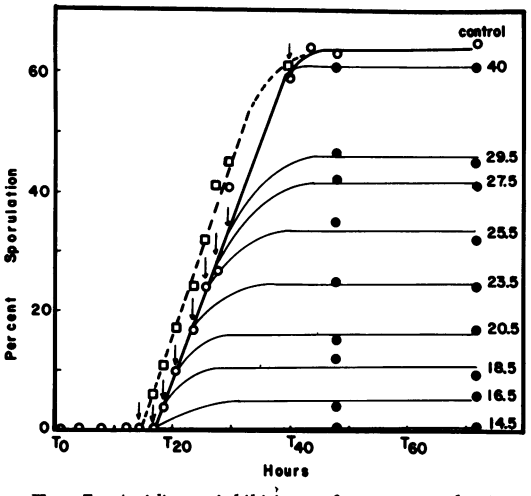

FIG. 7. *Actidione inhibition of ascus production. The arrows indicate time of addition of 50 μg of actidione per ml to a sporulating culture of Z140-9A. The numbers at the right of each line indicate the time at which actidione was added (addition from 0 to 14.5 hr gave cultures with 0% asci at 72 hr). The dotted line indicates the capacity to sporulate after actidione addition (i.e., final percentage of asci at 72 hr plotted on the ordinate, time of addition of actidione on the abscissa).*

T_{72} in cultures exposed to actidione (at the times indicated by the arrows) is indicated by the dashed line in Fig. 7. Continued ascus formation after the addition of actidione is an index of the fraction of cells that had already completed the protein synthesis required for ascus formation at the time actidione was added.

DISCUSSION

Studies on meiosis in yeast reported here and elsewhere (6, 7) lead to the suggestion that sporulation is dependent upon acetate metabolism and on the physiological age of the cells when transferred to sporulation medium. The experiments reported here demonstrate that, when cells are transferred to sporulation medium at the optimal period of growth for sporulation, evolution of ^{14}C-CO_2 from acetate oxidation proceeds without any apparent lag. These results indicate that glucose-grown cells at the appropriate physiological age for sporulation readily respire acetate. The data are in agreement with previous studies of the initial Q_{O_2} of cells transferred to acetate sporulation medium, which suggested that sporulation competency is dependent in part upon the development of the oxidative ability which appears during the latter stages of growth in glucose medium (6-8). Further evidence in behalf of this interpretation comes from the observation that logarithmic-phase cells grown in acetate nutrient medium display sporulation competency upon transfer to sporulation medium, whereas logarithmic-phase glucose-grown cells of the same strains sporulate slowly or not at all (14).

The incorporation of acetate-2-^{14}C carbon into macromolecular fractions indicates that the acetate consumed is an important source of metabolites for the biosynthetic reactions which occur during sporulation. At T_{50}, when ascus development is essentially complete, 62% of the acetate consumed has been respired as CO_2, 22% is present in the soluble pool, and 16% has been incorporated into macromolecules or cellular debris. The residue of cellular debris which remains after extraction of lipids, nucleic acids and proteins retains approximately 21% of the acetate incorporated into cells. The nature of this material remains to be elucidated.

Meiosis and sporulation in yeast, like sporulation in bacteria (1, 9, 11, 15), is associated with extensive protein turnover. As shown here, the protein content of sporulating cells first increases and then declines as sporulation proceeds. Experiments with ^{3}H-lysine-labeled cells (Fig. 6) clearly demonstrate that the fluctuations in protein content are a combination of protein synthesis and turnover. Synthesis is continuous throughout sporulation and shows two maxima, one before asci appear (T_6) and a second during ascospore development (T_{28}). The observation (Fig. 7) that the inhibition of protein synthesis at any time before the completion of ascus development reduces the frequency of asci produced indicates that the protein synthesis observed is necessary for sporulation. These data suggest that the proteins required are continually produced in individual cells until the last stages of ascospore assembly. Further experiments are needed to test this hypothesis and to determine whether the essential gene products are produced in an ordered or random sequence.

The physiological changes (effect of physiological age on the capacity to sporulate and the change in DNA, RNA, protein, and dry weight) which accompany meiosis and ascus formation of homothallic diploids of *Saccharomyces* are similar to the events which have been described for heterothallic diploids (5–7). Thus, the use of homothallic diploids which facilitate study of the genetic control of meiosis do not appear to introduce additional complexities into this system.

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