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Proteinase Activities of *Saccharomyces cerevisiae* During Sporulation

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Sporulation in *Saccharomyces cerevisiae* occurs in the absence of an exogenous nitrogen source. Thus, the internal amino acid pool and the supply of nitrogen compounds from protein and nucleic acid turnover must be sufficient for new protein synthesis. Since sporulation involves an increased rate of protein turnover, an investigation was conducted of the changes in the specific activity of various proteinases. A minimum of 30% of the vegetative proteins was turned over during the course of sporulation. There was a 10- to 25-fold increase in specific activity of various proteinases, with a maximum activity around 20 h after transfer into the sporulation medium. The increase in activities was due to de novo synthesis since inhibition of protein synthesis by cycloheximide blocks both an increase in proteinase activities and sporulation. There was no increase observed in proteinase activities of nonsporogenic cultures (*a* and *α/α* strains) inoculated into the sporulation medium, suggesting that the increase in proteinase activities is "sporulation specific" and not a consequence of step-down conditions. The elution patterns through diethylaminoethyl-Sephadex chromatography of various proteinases extracted from T_0 and T_{18} cells were similar, and no new species was observed.

Sporulation in yeast is complex, involving meiosis and subsequent ascospore formation. Yeast, therefore, provides a convenient system for studying developmental controls because of its single-cell nature and fairly well-known genetics. *Saccharomyces cerevisiae* is induced to sporulate by transferring it to a potassium acetate medium free from nitrogen metabolites (7, 9). It has been well established that nitrogen metabolites in the sporulation medium suppress sporulation (29). Sporulation requires protein synthesis; addition of cycloheximide inhibits the process (9, 16). Since sporulation occurs in the absence of an exogenous nitrogen source, the internal amino acid pool and the supply of nitrogen compounds from protein turnover must be sufficient for new protein synthesis (9). This observation initiated the present interest in studying the proteinases and protein turnover during sporulation in yeast.

Yeast cells produce a number of different proteolytic activities (14, 15, 21), and little is known about their physiological function (31). Although intracellular protein turnover has long been known to increase in resting cells (12), information on protein turnover during sporulation in yeast has been obtained only recently (9, 16). At present, the only published information available on the proteinase activities during sporulation in yeast is that of Chen and Miller (6), who observed an increase in acid proteinase (proteinase A) activity of whole cells during development.

In this communication, we report the time sequence of the formation of three proteinases during sporulation. The results show that the increase in specific activity of proteinases A, B, and C during sporulation is due to de novo synthesis. This increase in activities seems to be specific for sporulation and not merely a consequence of step-down conditions. No new species of proteinase specific for sporulation were observed.

MATERIALS AND METHODS

Yeast strains. The yeast strains used were: Y289, $\frac{a\ HO\ HM\alpha\ HM\alpha\ arg\text{-}4\text{-}1}{\alpha\ HO\ HM\alpha\ HM\alpha\ arg\text{-}4\text{-}1}$; Y111, $\frac{a}{\alpha}\frac{met}{met}$; and Y185a, *a gal-2*.

(The symbols are as follows: *a* and *α*, mating type alleles; *arg*, arginine auxotroph; *met*, methionine auxotroph; *gal*, galactose nonfermenters; HO and HM, genes controlling homothallism [38].)

Culture media and growth conditions. The following media were used: yeast extract peptone dextrose (2% dextrose, 2% [Difco] peptone, and 1% yeast extract); yeast extract peptone galactose medium

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containing 2% galactose instead of dextrose; and sporulation medium containing 2% potassium acetate and 10 to 20 μg of auxotrophic requirements per ml as indicated. Cultures to be sporulated were inoculated into yeast extract peptone dextrose medium at a cell density of 5×10^4 cells/ml and incubated with shaking at 30 C. The cells were harvested by centrifugation after 25 h of growth to early stationary phase, washed twice with sterile, distilled water, suspended in sporulation medium at a density of 5×10^7 cells/ml, and incubated with shaking at 25 C. The percentage of asci in sporulating cultures was determined by counting at least 500 cells under the phase-contrast microscope.

Cell extract preparation and enzyme assay. Cells were harvested, washed three times with water suspended in potassium phosphate buffer (0.01 M, pH 7.0), and disrupted by passage through a chilled French pressure cell at 6,000 lb/in². The breakage of the cells was consistently over 90%. Cell debris was sedimented by centrifuging the extract at 27,000 × *g* for 30 min in a Sorvall RC2B centrifuge and then at 45,000 rpm for 90 min in a Ti50 rotor in a Beckman L2 centrifuge. All manipulations were carried out at 0 to 4 C.

Proteinase A was assayed at pH 3.0, with acid-denatured hemoglobin (Nutritional Biochemicals Corp., Cleveland, Ohio) as the substrate, by the method of Hayashi et al. (14). Prior to enzymatic assay, the enzyme preparation was incubated with 1.7 mM phenylmethyl sulfonylfluoride (Schwarz/Mann, Orangeburg, N.Y.) to inhibit the proteinase B and C present in the sample. One unit of proteinase A was defined as the amount of enzyme that gave an absorbancy at 280 nm equivalent to 1 μg of L-tyrosine in a 1-min reaction at 30 C. Proteinase B was assayed by the method of Lenney and Dalbec (19) with casein or azocoll as the substrate (33). One unit of proteinase B was defined as the quantity that caused an increase in optical density at 520 nm of 1.0 per 30 min. Proteinase C activity was determined by the method of Hayashi et al. (14) with carbobenzoxy-L-glutamyl-L-tyrosine (Sigma Chemical Co., St. Louis, Mo.) as the substrate. One unit of proteinase C was defined as the quantity that caused an increase in optical density at 570 nm of 1.0 per 30 min. Uridine diphosphogalactose–4-epimerase (EC 5.1.3.2) was assayed by the method of Klar and Halvorson (18). One unit of epimerase was defined as the amount that caused an increase in optical density at 340 nm of 0.001 per min at 30 C. All enzymatic activities were assayed at 30 C.

The proteinases in the cell extracts were activated by adjusting the pH to 5.0 with 3 N acetic acid and incubating at 15 C for 20 h by the method of Lenney et al. (21).

For diethylaminoethyl-Sephadex chromatography, cells from 1,000-ml cultures were broken in 0.01 M tris(hydroxymethyl)aminomethane buffer (pH 7.0), and extracts were prepared and activated as described above. The activated extract was dialyzed against five changes of buffer and loaded on a diethylaminoethyl-Sephadex A-25 column equilibrated with the same buffer. After the column was washed, the proteinases were eluted with a linear 0.0 to 0.4 M sodium chloride gradient.

Units and assay for inhibitor B. A partially purified yeast proteinase preparation was assayed with and without added inhibitor. The inhibitor was obtained by heating the cell-free extract for 10 min in boiling water. The denatured proteins were removed by centrifugation, and the supernatant fluid was analyzed for inhibitor (1, 20, 34). One unit of inhibitor was defined as the amount that decreases proteinase activity by 1 unit.

Assay for proteins. Protein concentration was assayed by the method of Lowry et al. (23), with bovine serum albumin as the standard.

RESULTS

Protein turnover during sporulation. Cells of Y289 were grown in yeast extract peptone dextrose medium containing L-[^{14}C]arginine to early stationary phase and then after washing were transferred to unlabeled sporulation medium. As shown in Fig. 1, the ratio of the trichloroacetic acid-precipitable counts at indicated times to the counts at T_0 (T_x/T_0) decreases steadily after a lag of about 4 h and reaches a plateau after T_{20}. It is clear that about 30% of the acid-precipitable [^{14}C]arginine counts decrease during the course of sporulation. This is the minimum estimate of the protein turnover since some of the radioactivity obtained by the degradation of the vegetatively labeled proteins was reincorporated. A similar but more pronounced increased turnover rate during sporulation was observed by H. Betz and H. Holzer (personal communication). The kinetics of sporulation are also shown in Fig. 1.

To study the inactivation of a specific protein

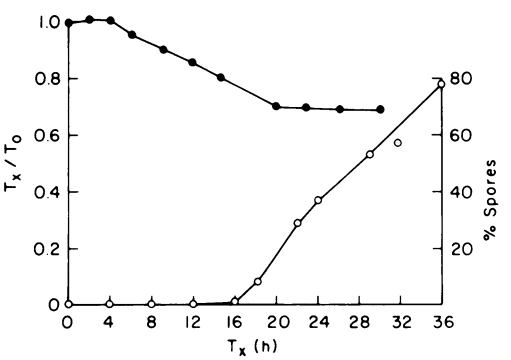

FIG. 1. *Protein turnover during sporulation of Y289 and kinetics of sporulation. The culture was grown in yeast extract peptone dextrose medium containing 0.2 μCi of* L-[^{14}C]*arginine per ml to early stationary phase, washed, and suspended in sporulation medium containing 100 μg of* L-*arginine per ml. The trichloroacetic acid-precipitable counts were measured initially (T_0) and at indicated 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. Symbols: ●, T_x/T_0; and ○, percentage of sporulation.*

during sporulation, the activity of uridine diphosphogalactose-4-epimerase was followed. As shown in Fig. 2, the epimerase activity decreases to about 4% in 12 h, and by T_{16} the activity is undetectable. This experiment suggests that, even though there is only a 30% decrease in the acid-precipitable counts (Fig. 1), specific proteins might be completely inactivated during sporulation.

Intracellular proteinase activities during sporulation. Assays for intracellular activities were made on crude cell-free extracts after breakage of cell suspensions. The specific activities of proteinase A, B, and C plotted in Fig. 3 reveal that these proteinase activities increase considerably during the course of sporulation.

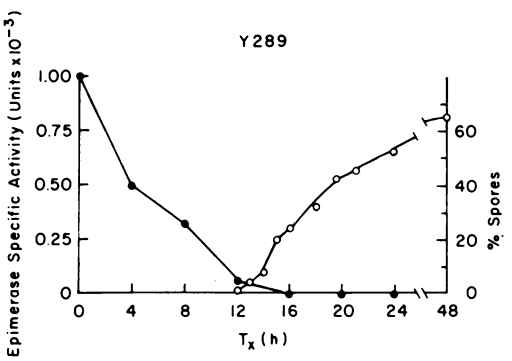

FIG. 2. *Kinetics of inactivation of uridine diphosphogalactose-4-epimerase during sporulation of Y289. Early stationary-phase cells grown in yeast extract peptone galactose medium were washed and inoculated into sporulation medium. At indicated intervals, 100 ml of sporulating cells was harvested, and the extracts made were assayed for epimerase activity. Symbols: ●, epimerase specific activity; and ○, percentage of sporulation.*

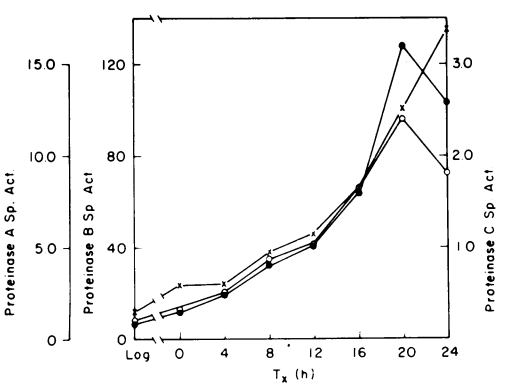

FIG. 3. *Proteinase activities during sporulation of Y289. Extracts were prepared and enzyme activities were assayed from 500-ml cultures harvested at indicated times during sporulation. Symbols: ●, proteinase A; ○, proteinase B; and X, proteinase C specific activity. Log, extract made from logarithmically growing cells.*

Proteinases A and B showed maximum activity at T_{20}, and proteinase C activity is still rising at T_{24}. The specific activities of all of these proteinases are lowest in extracts made from logarithmically growing cells.

To determine whether the increase in the specific activity of these proteinases was due to de novo synthesis, protein synthesis was inhibited by cycloheximide (Upjohn Co., Kalamazoo, Mich.). As is clear from Fig. 4, there is no increase in the activities of proteinases once protein synthesis is inhibited by cycloheximide. Addition of cycloheximide at T_0 (not shown) completely inhibits sporulation (9, 24).

Intracellular activities of nonsporulating strains inoculated into sporulation medium. Since sporulation occurs under starvation conditions, the increase in the specific activities of the proteinases might result from the stepdown conditions and might not be connected with the sporulation process itself. This possibility was studied in a haploid and diploid strain homozygous for the sex locus incapable of sporulation (32). Figure 5 shows there is insignificant increase in the activities of the proteinases strain in Y185a (haploid) and strain Y111 (a/α, diploid) when inoculated into sporulation medium. These experiments suggest that the increase in specific activity in sporulation medium is observed only in the sporulating strains and that this increase is not a consequence of starvation conditions.

Kinetics of proteinase activation. Proteinases obtained from yeast have been known to increase in activity in crude extracts by various treatments, including incubation at pH 5.0 (13, 15, 17, 20). Therefore, these proteinases were activated by incubation of the crude extracts at pH 5.0 (Table 1, experiments 1 and 2). There is an increase in activity of proteinase A by about 70% and of proteinase B and C by about three-

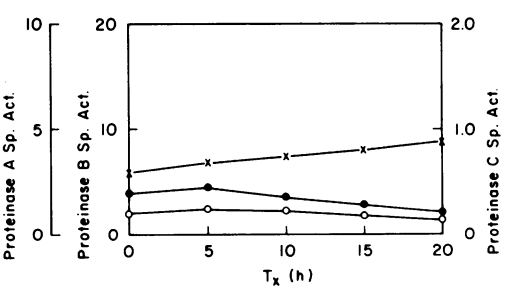

FIG. 4. *Effect of cycloheximide on the appearance of proteinase activities of Y289 during sporulation. Cycloheximide (100 μg/ml) was added at T_0. A 0.500-ml portion of cells was harvested at indicated times and assayed for proteinase activities. Symbols: ●, proteinase A; ○, proteinase B; and X, proteinase C specific activity.*

fold. Also, when boiled crude extract is added to the activated extract, there is a decrease in the activity of the incubated extract for proteinases A and B but not for proteinase C (experiment 4). Experiment 4 suggests the presence of heat-stable inhibitors of proteinase A and B in the crude extract. Recently, specific proteinase inhibitors have been purified from yeast (1, 2, 10, 25, 34, 39). The addition of a boiled, incubated extract to the incubated extract (experiment 5) does not decrease the activity of the incubated extract, indicating that the incubation at pH 5.0 inactivates the proteinase inhibitors. Figure 6 shows the kinetics of activation of proteinases A, B, and C present in the extract made from T_{18} cells by adjusting the pH to 5.0 and incubating it at 25 C. There is a 70% increase in activity of proteinase A, about a 2.5-fold increase for proteinase C by 10 h, and about a threefold increase for proteinase B by 5 h of incubation. During the incubation time, the total protein content of the extract decreases.

Is the increase in activity of proteinases during sporulation due to the inactivation of their corresponding inhibitors? Since there is a severalfold increase in activity of various proteinases by pH 5.0 treatment, the increase in specific activities during sporulation might be a result of their activation; i.e., there might be a "sporulation-specific" activation process that appears during the course of sporulation. If this is true, one would expect that proteinase activities during sporulation might be mainly present as active species. This possibility was investigated by studying the activation of various samples obtained during the course of sporulation. As shown in Fig. 7 for proteinase A, Fig. 8 for proteinase B, and Fig. 9 for proteinase C, the activation of all three enzymes occurs to approximately the same extent in all samples. These results suggest that there is no sporulation-specific activation process appearing during sporulation since all of the samples obtained during sporulation are activated to the same extent, i.e., the ratio of active to inactive activity remains constant.

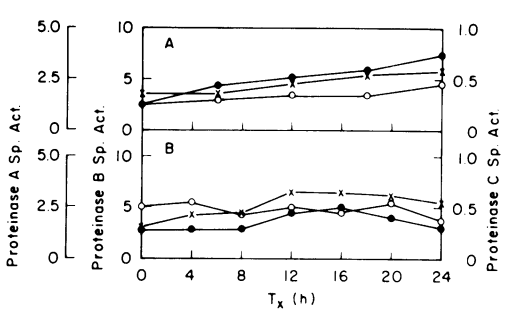

FIG. 5. *Proteinase activities of (A) Y185a (haploid) and (B) Y111 (a/α diploid) inoculated into sporulation medium. A 0.500-ml portion of cells was harvested at indicated times and assayed for proteinase activities. Symbols: ●, proteinase A; ○, proteinase B; and X, C specific activity.*

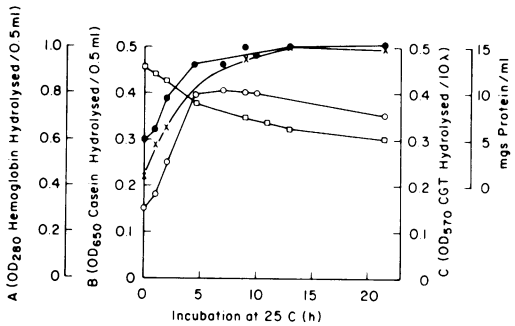

FIG. 6. *Kinetics of activation of proteinases. Extract made from sporulating Y289 cells (T_{18}) was adjusted to pH 5.0 with 3 N acetic acid and incubated at 25 C. At indicated times samples were obtained and assayed for proteinase activities. Symbols: ●, proteinase A; ○, proteinase B; X, proteinase C activity; and □, protein concentration.*

TABLE 1. *Factors affecting proteinase activities*

Experiment	Treatment	Activity units[a]		
		Proteinase A	Proteinase B	Proteinase C
1	Crude extract[b]	4.7	0.20	15
2	Incubated extract[c]	8.2	0.64	46
3	Incubated extract + crude extract	13.4	0.83	60
4	Incubated extract + boiled crude extract[d]	5.9	0.26	44
5	Incubated extract + boiled incubated extract	8.0	0.62	43
6	Boiled crude extract or boiled incubated extract	0	0	0

[a] For experiments 1, 2, and 6 only, 0.5 ml of each sample was assayed and recorded. For experiments 3, 4, and 5, 0.5 ml of each sample was mixed, and the total activity in the mixed sample was recorded.

[b] Crude extract was prepared from sporulating Y289 cells harvested at T_4.

[c] Incubated extract was prepared by incubation of crude extract at pH 5.0 and 15 C.

[d] Boiled extract was prepared by boiling crude extract for 10 min followed by centrifugation to remove precipitated proteins.

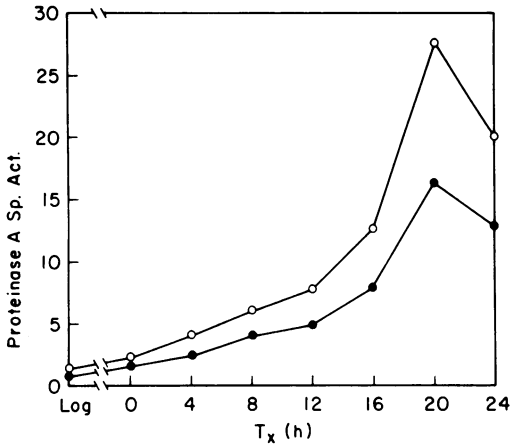

FIG. 7. *Activation of proteinase A in cell-free extracts made from sporulating Y289 cells harvested at indicated times. Activation was carried out by adjusting the pH of the extract of 5.0 and incubating it at 15 C for 20 h. Proteinase A activity was assayed before and after activation. Symbols: ●, unactivated; and ○, activated extract activity.*

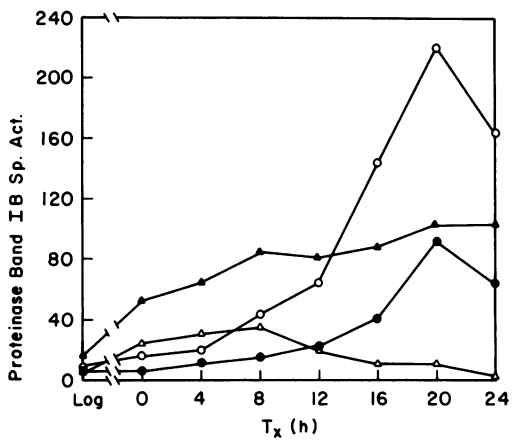

FIG. 8. *Activation of proteinase B in cell-free extract made from sporulating Y289 cells. Proteinase B activity and its inhibitor was assayed before and after activation of the extract. Symbols: ●, unactivated; ○, activated extract proteinase B activity; ▲, inhibitor from unactivated extract; and △, inhibitor from activated extracts.*

The results in Table 1 show the existence of heat-stable proteinase A and B inhibitors in yeast. To determine whether the activation of proteinase B was a result of the inactivation of its inhibitor, the level of proteinase B inhibitor was assayed in both unactivated and activated samples. The activated samples have lower levels of the inhibiton (Fig. 8). The increase in the activity during activation is coincident with the decrease in the level of the inhibitor, suggesting that the activation is due to the inactivation

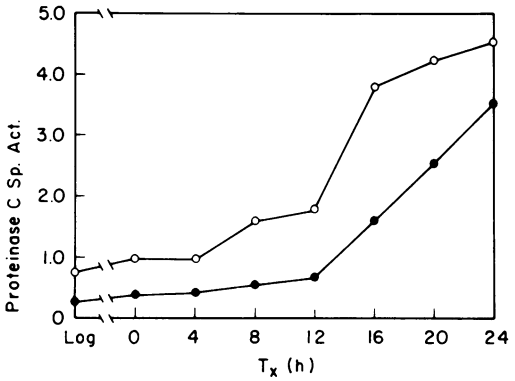

FIG. 9. *Activation of proteinase C in extract made from sporulation Y289 cells. Proteinase C activity was assayed before and after activation. Symbols: ●, unactivated; and ○, activated extract proteinase C activity.*

of the inhibitor.

Elution patterns of proteinase activities extracted for T_0 and T_{18} cells. The increase in protein turnover during sporulation might be due to the appearance of a new species of proteinase activity. To investigate this possibility we compared the elution patterns of the proteinase activities extracted from T_0 and T_{18} cells. As shown in Fig. 10A and B, the elution pattern of various proteinase activities is similar on diethylaminoethyl-Sephadex chromatography. No new species appeared during sporulation. However, under these conditions, any new proteinase with different substrate specificity of conditions will not be detected since we are assaying fractions only against standard substrates and conditions for the known proteinases.

DISCUSSION

The purpose of these experiments was to study the proteolytic activity of sporulating yeast cells. The present study provides evidence that, when vegetative cells are induced to sporulate, increases in specific activity of proteinases A, B, and C occur. These increases in activities appear to be only in sporulating cells, suggesting it is a sporulation-specific phenomenon and not merely a consequence of step-down conditions. Also, the increase in activities appears to be due to de novo synthesis since cycloheximide stops this increase in sporulating cells. We failed to observe any change in the elution pattern of various proteinases extracted from T_0 and T_{18} cells on diethylaminoethyl-Sephadex chromatography, and no new species was observed during sporulation of yeast.

The increase in proteolytic activity during sporulation might lead to the increased rate of turnover of proteins during this process. Our

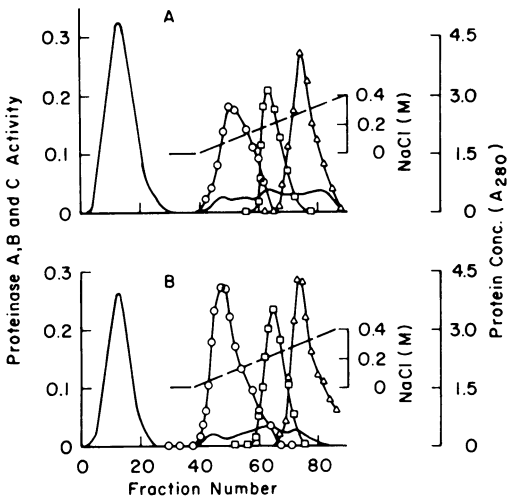

FIG. 10. *Elution of proteinases through diethylaminoethyl-Sephadex chromatography. Activated extract of (A) T_0 or (B) T_{18} cells was applied to 20-ml diethylaminoethyl-Sephadex column. The column was washed with 75 ml of tris(hydroxymethyl)-aminomethane buffer (0.01 M, pH 7.0), and the proteinases were eluted with a linear gradient of 75 ml of 0.0 M and 75 ml of 0.4 M sodium chloride. Samples (3-ml) were collected. A 100-μl portion of each fraction was assayed for proteinase activities, and the increase in optical density at 280 nm for proteinase A (□), 520 nm for proteinase B (○) by the Azocoll assay method, and 650 nm for proteinase C (△) in the 30-min assay time is plotted. (----), Sodium chloride concentration; (—), protein concentration.*

data suggest that the total inactivation of uridine 5'-diphosphate epimerase far exceeds the net protein breakdown (Fig. 1 and 2). However, we cannot establish that epimerase inactivation is due to its breakdown. In a previous report (A. J. S. Klar, A. Cohen, and H. D. Halvorson, submitted for publication, we observed no change in the ribonucleic acid polymerase forms extracted from sporulating cells. Thus, in spite of high protein turnover during sporogenesis, there seems to be selectivity for inactivation of individual proteins. These findings might be attributed to the inherent susceptibility of various proteins to degradation by proteinases or to compartmentation of proteinases or to compartmentation of proteinases and specific proteins in vivo. Setlow (35) observed that 20% of the proteins originally present in *Bacillus megaterium* spores degraded in the first 20 min of germination are mainly due to the degradation of two low-molecular-weight basic proteins, both of which are highly sensitive to proteolysis in vitro. Individual proteins are known to vary in their sensitivity to various proteases (3). Bukhari and Zipser (4) were successful in isolating mutants of *Escherichia coli* defective in the degradation of nonsense fragments of β-galactosidase. At least some of these *deg* (degradation) mutants were allelic to the *lon* locus (36). The *lon* mutants are presumed to be altered in the cell membrane (22). One of the possible explanations for these results is that the selective degradation of the defective protein fragments is mediated by their selective transport through the vacuolar membrane to the proteases in the vacuoles. In *S. cerevisiae*, various proteinases have been observed to be localized in vacuoles (5, 21, 28), and their corresponding inhibitors are present in the extravacuolar cytosol (21, 27, 39). During sporulation in yeast, there is an increase in vacuolization (30), and finally the vacuolar membranes break down (37) as the sporulation progresses. This breakdown of the vacuoles might function to initiate protein breakdown in sporulating cells.

A protein turnover rate between 1 and 18%/h has been reported in different species of *Bacillus* during sporulation (8). This process must play a key role in furnishing amino acids to a system that is undergoing differentiation in a medium devoid of the usual carbohydrates and amino acids. Mandelstam (26) has pointed out that protein catabolism may increase in poor environments enabling cells to synthesize new enzymes appropriate to the new environment. The induction of β-galactosidase by a gratuitous inducer was inhibited by the addition of serine protease inhibitor, phenylmethyl sulfonylflouride, in nitrogen-starved *E. coli* cells, presumably because of the limitation of amino acids (11).

The autoradiogram patterns (i.e., newly synthesized proteins) of vegetative yeast cells differ quite remarkably from those obtained from sporulating cells (16). Many vegetative proteins are not synthesized and, conversely, a number of new protein bands appear after transfer to sporulation medium. It remains to be seen whether these increased proteinase activities observed during sporulation are required to provide amino acids and/or an energy source for biosynthesis of these new proteins or whether they have some specific function, i.e., activation of some sporulation-specific proteins. The exact role of proteinase activities during sporulation will be clear from the study of various proteinase-negative strains. Attempts are in progress in this laboratory to isolate such mutants.

ACKNOWLEDGMENTS

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