PROTEIN DEGRADATION, MEIOSIS AND SPORULATION IN PROTEINASE-DEFICIENT MUTANTS OF *SACCHAROMYCES CEREVISIAE*

GEORGE S. ZUBENKO AND ELIZABETH W. JONES

Department of Biological Sciences, Mellon Institute, Carnegie-Mellon University, Pittsburgh, PA 15213

Manuscript received September 12, 1980
Revised copy received December 9, 1980

ABSTRACT

During the process of sporulation, **a**/α diploids degrade about 50% of their vegetative proteins. This degradation is not sporulation specific, for asporogenous diploids of **a**/**a** mating type degrade their vegetative proteins in a fashion similar to that of their **a**/α counterparts. Diploids lacking carboxypeptidase Y activity, *prc1/prc1*, show about 80% of wild-type levels of protein degradation, but are unimpaired in the production of normal asci. Diploids lacking proteinase B activity, *prb1/prb1*, show about 50% of wild-type levels of protein degradation. The effect on degradation of the proteinase B deficiency is epistatic to the degradation deficit attributable to the carboxypeptidase Y deficiency. The *prb1* homozygotes undergo meiosis and produce spores, but the asci and, possibly, the spores are abnormal. Diploids homozygous for the pleiotropic *pep4-3* mutation show only 30% of the wild-type levels of degradation when exposed to a sporulation regimen, and do not undergo meiosis or sporulation. Neither proteinase B nor carboxypeptidase Y is necessary for germination of spores.——Approximately half of the colonies arising from **a**/**a** or α/α diploids exposed to the sporulation regimen that express an initially heterozygous drug-resistance marker (*can1*) appear to arise from mating-type switches followed by meiosis and sporulation.

EXTENSIVE protein degradation and turnover occur when diploid yeast cells of **a**/α mating type are induced to undergo meiosis and sporulation by transfer to an acetate-containing medium free of nitrogen (ESPOSITO *et al.* 1969; HOPPER *et al.* 1974; KLAR and HALVORSON 1975; BETZ and WEISER 1976). That this protein degradation might be sporulation specific was suggested by HOPPER *et al.* (1974), since **a**/**a** or α/α diploids showed 0–10% degradation compared to the 25–30% seen in **a**/α diploids. However, BETZ and WEISER (1976) reported that the pattern and extent of protein degradation was nearly the same for a haploid of **a** mating type as it was for the **a**/α diploid.

That the vacuolar proteinases A, B and carboxypeptidase Y (CABIB, ULANE and BOWERS 1973; HASILIK, MÜLLER and HOLZER 1974; LENNEY *et al.* 1974; MATERN, BETZ and HOLZER 1974; WIEMKEN, SCHELLENBERG and URECH 1979) might be responsible for the observed protein degradation and might be essential for sporulation was suggested by BETZ and WEISER (1976), for the levels of these

three proteinases rise when the cells sporulate (CHEN and MILLER 1968; KLAR and HALVORSON 1975; BETZ and WEISER 1976) and no new proteinases are detected during sporulation (KLAR and HALVORSON 1975; BETZ and WEISER 1976).

Mutants deficient in one or more of these three proteinases have been isolated (WOLF and FINK 1975; BETZ 1975, 1979; JONES, LUND and ZUBENKO 1975; JONES 1977; MITCHELL and JONES 1977; ZUBENKO, MITCHELL and JONES 1978, 1979a, b; WOLF and EHMAN 1978; WOLF, BECK and EHMAN 1979). Structural genes for proteinases B, *prb1*, (ZUBENKO, MITCHELL and JONES 1978; 1979a, b) and for proteinase C (carboxypeptidase Y), *prc1* (WOLF and WEISER 1977), have been identified.

We report here investigations of protein degradation, meiosis and sporulation in strains deficient in one or more proteinases as a consequence of the mutations they bear.

Strains and media: The *prb1–9* allele is an amber mutation in the structural gene for proteinase B (ZUBENKO, MITCHELL and JONES 1979b, 1980). The *prb1–1122* mutation was isolated in a *trp1* derivative of X2180–1B and is an ochre mutation. The mutants bearing the *prc1–125*, *prc1–36* or *pep4–3* alleles were isolated in the *trp1* derivative of X2180–1B as clones unable to cleave acetylphenylalanine β-naphthyl ester, using the procedure described by JONES (1977). The two *prc1* alleles lie in what is thought to be the structural gene for proteinase C (carboxypeptidase Y) (WOLF and WEISER 1977). The *pep4–3* mutation, allelic to *pep4–1* is a pleiotropic mutation that lowers the levels of proteinases A, B and carboxypeptidase Y to 10%, 7% and 3%, respectively, of the corresponding wild-type activities, and reduces the activities of ribonuclease and alkaline phosphatase to about 8 and 25%, respectively, of the wild-type levels (HEMMINGS, ZUBENKO and JONES 1980; JONES *et al.* 1980). The repressible alkaline phosphatase, which cleaves α-naphthyl phosphate, appears to be missing in the *pep4–3* mutant (unpublished observations). Mutations in *CAN 1* were isolated, where relevant, by selection on medium containing canavanine sulfate.

Ten diploid strains were constructed for the experiments on protein degradation. Constructions involved mating a strain of the M16–14C background to one of the X2180–1B genetic background. After isolation of the diploids, the relevant markers were recovered in homozygous condition by isolation of the appropriate mitotic recombinants. A minimum of two and a maximum of four successive mitotic recombinational events were required to generate the 10 diploids; all 10 were heterozygous for *leu1–1* and *ser1–171* and homozygous for *his5* and *can1*. Five different proteinase genotypes were employed. Diploids were homozygous for *prc1–125*, *prb1–9*, *prb1–9* and *prc1–125*, *pep4–3*, or wild-type alleles for relevant genes. Each proteinase genotype was isolated as an **a**/α and **a**/**a** diploid. The 10 diploids are isogenic except for the chromosome arms involved in the mitotic recombinational events.

Ten related diploids were constructed for the genetic experiments. All were heterozygous for *leu1–1*, *ser1–171* and *can1*. Genotypes for alleles affecting proteinase levels were +/+, *prc1–125/prc1–125*, *pep4–3/pep4–3*, *prb1–9/prb1–1122* and *prb1–9 prc1–36/prb1–1122 prc1–36*. The *prb1* and *prb1 prc1* homozy-

gotes were heterozygous for *his5*; the remaining diploids were homozygous for *his5*. Each of the proteinase genotypes was isolated in **a**/**a** and **a**/α mating types. Strain constructions involved an initial mating, followed by isolation of mitotic recombinants where necessary.

YEPD, SC, omission media, and canavanine containing medium were previously described (JONES and LAM 1973; ZUBENKO, MITCHELL and JONES 1979b). Presporulation growth medium (PSP phthalate) was that of ROTH and HALVORSON (1969) for the protein degradation experiments. For some of the genetic experiments, the presporulation medium contained (per liter of distilled water) 10g potassium acetate, 8g nutrient broth and 10g yeast extract (PSP broth). Sporulation medium contained 3 mM L-leucine and 10g/l potassium acetate adjusted to pH 7 with acetic acid.

Sporulation and protein degradation: Cells were grown overnight in PSP broth. An 0.02 ml aliquot of culture was transferred into 25 ml of PSP phthalate medium containing 1 μC/ml of ^{3}H-leucine (50–60 Ci/mmole) in a 250 ml Erlenmeyer flask. After 24 hr of incubation at 30° in a gyrotory shaker, the cells were harvested by centrifugation for 10 min at 12,000 × g in a Sorvall RC5 centrifuge. The pellets were washed with sporulation medium and repelleted. The cells were resuspended in sporulation medium in 250 ml flasks at a cell density of about 2×10^{7}/ml (final volume of the culture was 15 to 25 ml). At the initiation of sporulation and at 8-hr intervals thereafter, 0.5 ml samples were removed and centrifuged 5 min at 1650 × g in a GLC-2 centrifuge. Fifty μl of medium was added to 10 ml Aquasol for scintillation counting, and 0.5 ml of 10% TCA was added to the remainder, which was vortexed. After 15 min, the suspension was filtered onto GFA filters. The filters were washed with 10% TCA and 95% ethanol, dried and counted, face up, in 10 ml Aquasol. At each time point, 50 μl of culture was added to Aquasol for counting, and a sample was diluted in water to allow determination of the percentage of asci by haemocytometer count of 200 to 300 cells. Analogous experiments were done using ^{35}S methionine. The cells were pregrown in PSP phthalate containing 2.7 μCi/ml of ^{35}S methionine (600 Ci/mmole). The sporulation medium contained 3 mM L-methionine.

Genetic events of sporulation: Cells were pregrown in PSP phthalate or PSP broth, harvested, washed with 0.9% NaCl and resuspended to about 2×10^{7} cells/ml in sporulation medium. At the beginning and at intervals thereafter, samples were removed and plated on arginine omission medium and on arginine omission medium to which canavanine sulfate had been added. The Petri plates were incubated at 30°. The ratio of the colony counts on the latter to the counts on the former provides an index of haploidization. At selected time points, Can^{R} colonies were picked and their nutritional requirements and mating types determined. Selected clones were crossed and their ploidies determined by segregational analysis of the meiotic products of these crosses.

At selected time points, samples were diluted 1/10 into 0.1 M dithiothreitol. After 20 min at room temperature, 1/20 volume of glusulase was added and the incubation was continued for one hr. Th samples were sonicated for 30 sec with a Branson sonifier equipped with a microtip at a power setting of 7. The cell sus-

pensions were diluted and plated on arginine omission medium and on canavanine medium.

Fluorescence microscopy: Meiotic cells were harvested and resuspended in 70% ethanol by vortexing. After at least 30 min, the cells were pelleted, washed with distilled water, resuspended in molten 0.5% agar containing one μg/ml of 4′,6-diamidino-2-phenylindole·2 HCl (DAPI), and incubated 30 min at 37°. DAPI stains nuclei (WILLIAMSON and FENNEL 1975). A drop of the suspension was placed on a slide and covered with a coverglass. The preparations were sealed and the agar allowed to solidify. Photomicrographs were made with a Zeiss photomicroscope III equipped with a III RS epifluorescence condenser. Illumination was provided by a XBO 75-W xenon arc with a Zeiss FITC filter. Tri-X film was developed in Diafine two-stage developer.

Reagents: L-[4,5-^{3}H(N)]-leucine, ^{35}S methionine and Aquasol were from New England Nuclear; DAPI from Accurate Chemical and Scientific Corporation, Hicksville, N.Y.; glusulase from Endo Laboratories, Inc., Garden City, N.Y.; dithiothreitol (DTT), canavanine sulfate and medium nutrilites were from Sigma; nutrient broth, yeast extract and Bacto-peptone were from Difco Laboratories. Standard reagents were from commercial sources.

RESULTS

Protein degradation during incubation in sporulation medium: Measurements of protein degradation were performed on cells that had been prelabelled during vegetative growth on ^{3}H-leucine. Protein degradation was determined by measuring the decrease in TCA precipitable radioactivity and by measuring the release of radioactivity into the medium after transfer to sporulation medium containing 3 mM leucine as a chase. Degradation patterns were determined for diploids of wild-type genotype, homozygous for a mutation in the proteinase C (carboxypeptidase Y) structural gene, *prc1–125*, homozygous for an amber mutation in the proteinase B structural gene, *prb1–9*, homozygous for both *prb1–9* and *prc1–125* or homozygous for *pep4–3*, a pleiotropic mutation that reduces the levels of proteinases A, B and C, as well as RNase and alkaline phosphatase. For all five proteinase genotypes, measurements were made on **a**/α and **a**/**a** diploids. In Figure 1, we report data on the release of radioactivity into the medium as a percentage of the radioactivity present in the culture for these 10 diploids. It is readily apparent that, for each of the five proteinase genotypes, protein degradation is the same for the **a**/α and **a**/**a** diploids. The rate of degradation begins to decline about the time that asci first appear in the cultures of the wild-type and *prc1*-bearing strains. As a comparable time, the rate of degradation also begins to decline in the strains bearing the *prb1* mutation, although normal asci are not seen in these cultures.

The data from Figure 1 for the five **a**/α diploids are compared in Figure 2. About 75% of the initial radioactivity is released into the medium by the wild-type cells. The carboxypeptidase-Y-deficient mutant shows a lower rate of release and eventually reaches a plateau value that is 88% of that seen for the wild type.

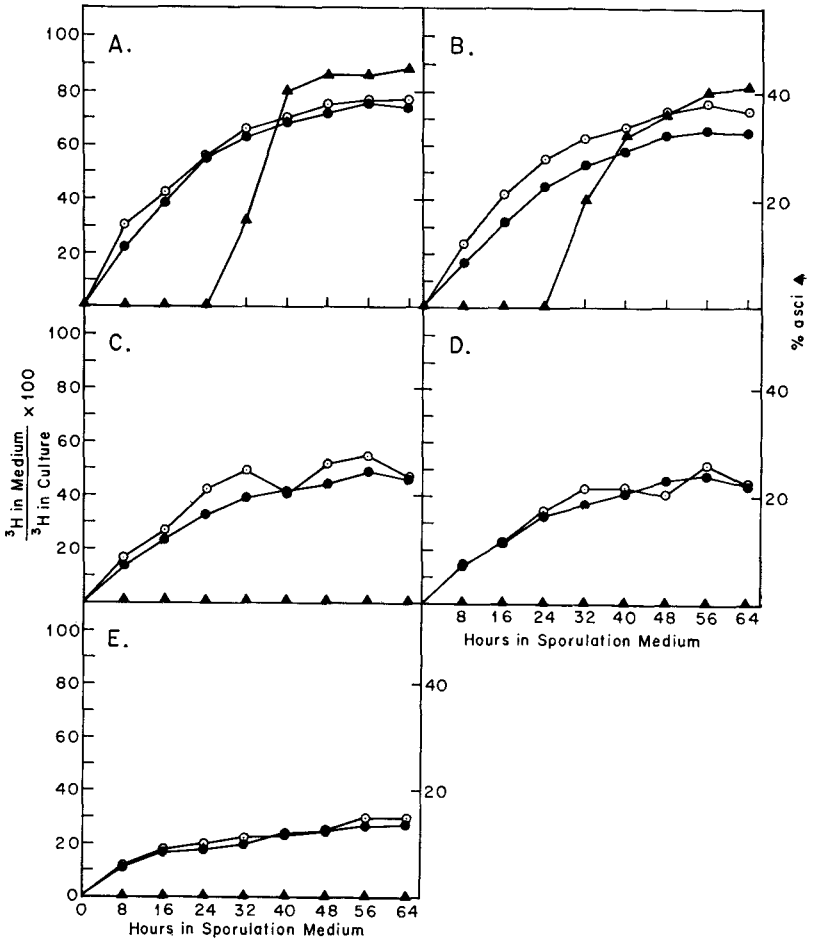

FIGURE 1.—Release of radioactivity into the medium by cells incubating in sporulation medium. Pregrowth and culture conditions and measurements of radioactivity released were performed as described in MATERIALS AND METHODS. (A) +/+; (B) *prc1-125/prc1-125*; (C) *prb1-9/prb1-9*; (4) *prb1-9 prc1-125/prb1-9 prc1-125*; (E) *pep4-3/pep4-3*. ⊙ **a/a** diploid; ● **a/α** diploid; ▲ % asci in **a/α** diploid. None of the **a/a** diploids produced asci.

The two strains lacking proteinase B by virtue of the *prb1-9* amber mutation show equivalent rates and plateau values for release of radioactivity. The plateau values are about 60% of that achieved by the wild-type strain, a substantial reduction. The observation that the patterns and plateau values are similar for the *prb1* and *prb1 prc1* strains suggests that proteinase B, a neutral endoproteinase, normally provides the substrate on which carboxypeptidase Y acts. This would not have been predicted, *a priori*, since proteinase A, also an endoproteinase, is present along with proteinases B and C in the yeast vacuole and might have been expected to contribute to the pool of proteinase C substrate. This is especially true since proteinase C exhibits little substrate specificity, *in vitro* (HAYASHI, AIBARA and HATA 1970; HAYASHI, MOORE and STEIN 1973). The pattern of degradation

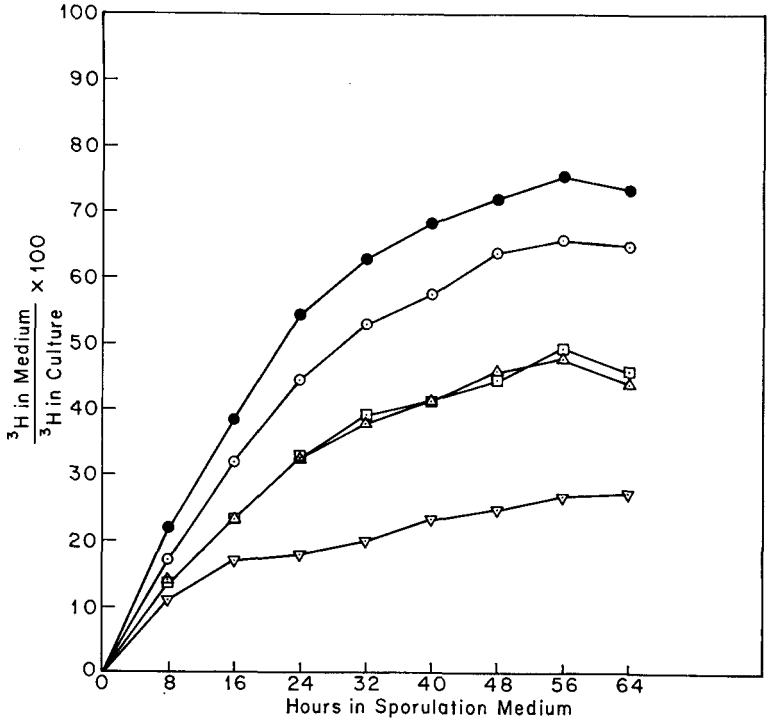

FIGURE 2.—Release of radioactivity into the medium by **a**/α diploids incubating in sporulation medium. Data from Figure 1. ●, +/+, ⊙, *prc1-125/prc1-125*; □, *prb1-9/prb1-9*; △, *prb1-9 prc1-125/prb1-9 prc1-125*; ▽, *pep4-3/pep4-3*.

seen for the strain bearing the pleiotropic *pep4-3* mutation differs substantially from that seen for the other strains. The initial rate of release of radioactivity is lower, and this release reaches a plateau much earlier. The final value is about 38% of that achieved by the wild-type strain. No asci are produced by the *pep4-3* homozygote.

The data presented in Figures 1 and 2 are not direct measures of protein degradation, for the release of radioactivity itno the medium must reflect degradation, the release of radioactivity from the pools and the rates of equilibration of the chase with the pools. A more direct measure of degradation, but one subject to much greater experimental error, is the release of radioactivity from acid-precipitable material. In Figure 3, we report loss of acid-precipitable radioactivity from cultures of wild-type diploids of **a**/α and **a**/**a** mating type. The **a**/α and **a**/**a** diploids degrade their protein with similar kinetics and to identical extents. The results are qualitatively similar to those presented in Figure 1 for the release of radioactivity into the medium. Possibly, the rate of loss of radioactivity from protein begins to fall somewhat in advance of the decline in rate of release of radioactivity into the medium. In Figure 4, we report the loss of acid-precipitable radioactivity for **a**/α diploids of all five proteinase genotypes. The wild-type diploid degrades about 49% of its proteins and the *prc1*-bearing mutant about

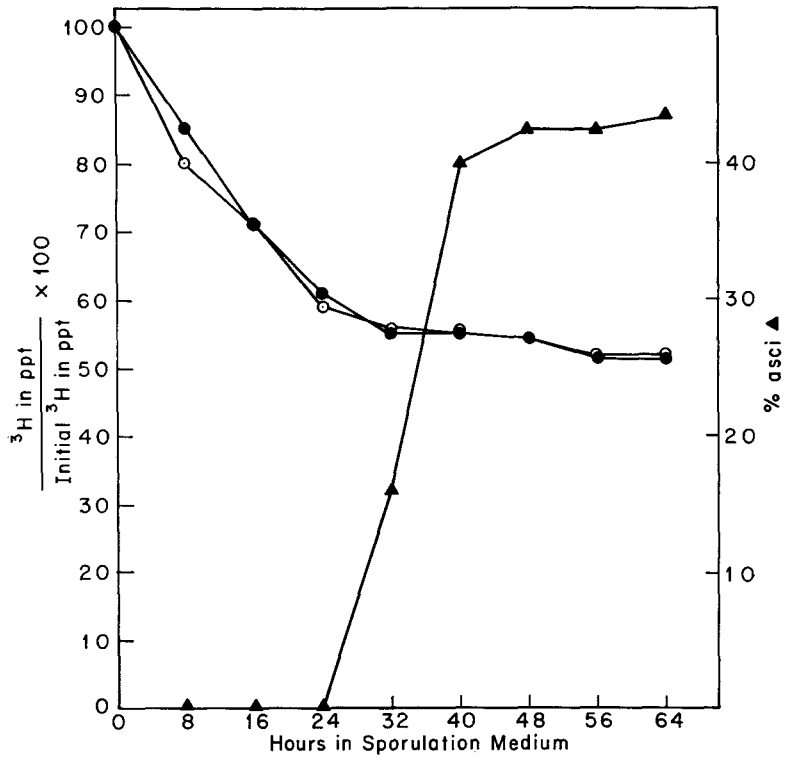

FIGURE 3.—Degradation of vegetative proteins by wild-type cells incubating in sporulation medium. Pregrowth and culture conditions and measurement of protein degradation were as described in MATERIALS AND METHODS. ⊙, a/a diploid; ▲, % asci.

38% (78% of wild type). The strains bearing *prb1–9* degrade about 25% of their protein (50% of wild type). Again, the data indicate that *prb1–9* is epistatic to *prc1–125* with respect to protein degradation. The *pep4–3* mutant diploid degrades about 15% of its acid-precipitable protein or about 30% of the extent of degradation seen in the wild-type diploid.

During the sporulation regimen, about 75% of the radioactivity present in the wild-type cells was eventually released into the medium, but only a 50% decrease of the radioactivity present initially in TCA- precipitable material was observed. The radioactivity present in TCA-soluble pools is released into the medium during sporulation and accounts for this difference (data not shown). The deficiency in protein degradation for the proteinase-deficient mutants measured as loss of acid-precipitable counts is somewhat greater than the deficiency measured as release of counts into the medium, but the results are qualitatively the same (a deficiency of 22% degradation as compared to a 12% deficiency for release of counts into the medium for the *prc1* diploid, for example).

In summary, the deficiency for proteinase B and/or carboxypeptidase Y results in decreased protein degradation. The *pep4–3* mutation drastically reduces the

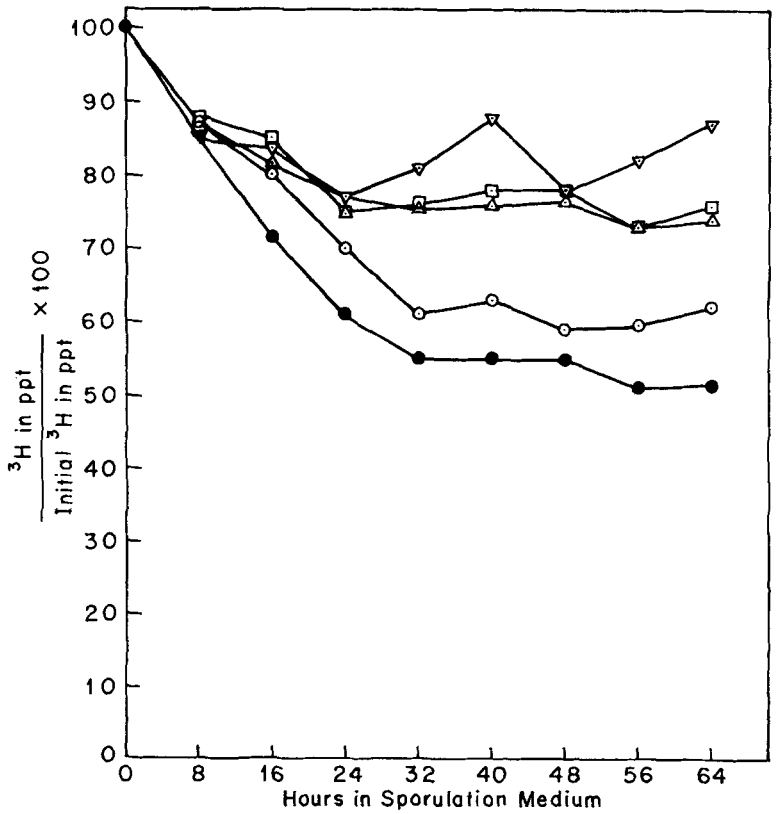

FIGURE 4.—Degradation of vegetative proteins by **a**/α diploids incubating in sporulation medium. Pregrowth and culture conditions and measurement of protein degradation were as described in MATERIALS AND METHODS. ●, +/+; ⊙, *prc1-125/prc1-125*; □,*prb1-9/prb1-9*; △, *prb1-9 prc1-125/prb1-9 prc1-125*; ▽, *pep4-3/pep4-3*.

rate and extent of protein degradation, but this result is difficult to interpret since the mutation is known to be pleiotropic.

Similar experiments were carried out on cells that had been prelabelled by growth in medium containing ^{35}S-methionine. Results very similar to those presented were obtained except that only 50% of the radioactivity was released into the medium in the wild type, rather than the 75% seen for labelled leucine (data not shown).

One can draw the following conclusions from the data presented in Figures 1–4: (1) The extensive protein degradation that occurs when cells are exposed to nitrogen-free sporulation medium is not sporulation specific, for nearly identical rates and extents of degradation are seen for **a**/α and **a**/**a** diploids of all five proteinase genotypes. The protein degradation seen is likely to be a response to nitrogen starvation, as it is also seen for vegetative cells starved of nitrogen (LOPEZ and GANCEDO 1979). (2) Protein degradation appears to be essential for normal sporulation, since normal asci are not seen in the *prb1* diploids or in *pep4-3* dip-

loids (however, see below). (3) Proteinase B and carboxypeptidase Y are responsible for about half the protein degradation that occurs when cells are exposed to nitrogen-free sporulation medium.

Cytological and genetical events of sporulation: As reported above, asci of normal appearance were not detected in strains homozygous for the *prb1* or *pep4–3* mutations. We reported previously that proteinase B activity is required for normal sporulation (ZUBENKO, MITCHELL and JONES 1978, 1979a, 1979b), because *prb1* homozygotes fail to sporulate, **a**/α diploids homozygous for temperature-sensitive *prb1* alleles produce asci at the permissive temperature (ZUBENKO, MITCHELL and JONES 1979a) and strains homozygous for *prb1*, but bearing at least one nonsense allele in *prb1* and heterozygous for an appropriate nonsense suppressor produce normal asci (ZUBENKO, MITCHELL and JONES 1980). That normal asci are not produced in *prb1* homozygotes is shown in Figure 5. The *prb1* homozygotes seem to have undergone at least some events of sporulation, for the cytoplasm appears to be subdivided into quadrants. The "sporulative" cells of the *prb1* homozygote are substantially smaller than the asci produced by wild-type cells.

To determine whether genetic events associated with meiosis were occurring, we constructed **a**/α and **a**/**a** diploids for the five proteinase genotypes used in the preceding experiments. These diploids were also made heterozygous for *can1*, a recessive mutation conferring resistance to canavanine. By determining whether the frequency of Can^R clones increased as a function of time in sporulation medium, we hoped to determine whether events of meiosis were occurring. As can be seen in Figure 6, all **a**/α diploids except the *pep4–3* homozygote gave rise to Can^R colonies at high frequencies. Moreover, the kinetics of production of Can^R colonies were similar for the four genotypes. The final level of Can^R colonies for the *prb1* homozygote is below that of the wild type in this experiment. In some experiments, however, the frequency was as high as 69% (see Table 1). The

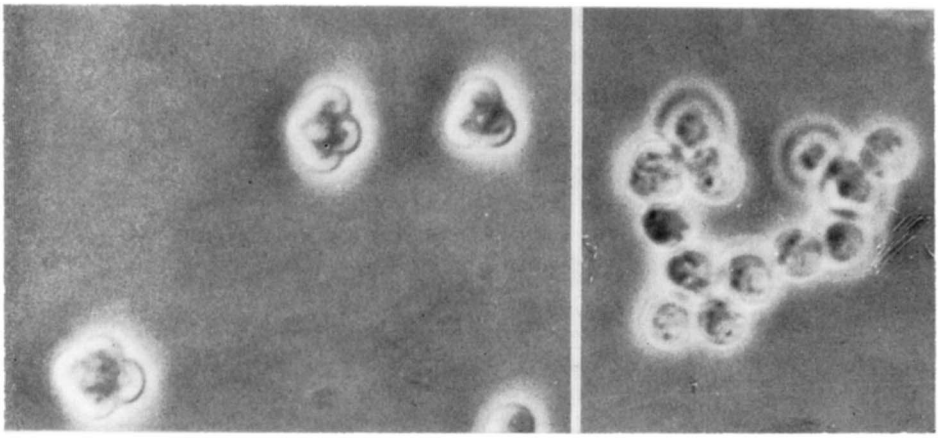

FIGURE 5.—Phase contrast micrographs of **a**/α diploids exposed to the sporulation regimen for 6 days. Cells were pregrown on PSP broth. +/+ homozygote is on the left; *prb1–9/prb1–1122* is on the right (× 1280).

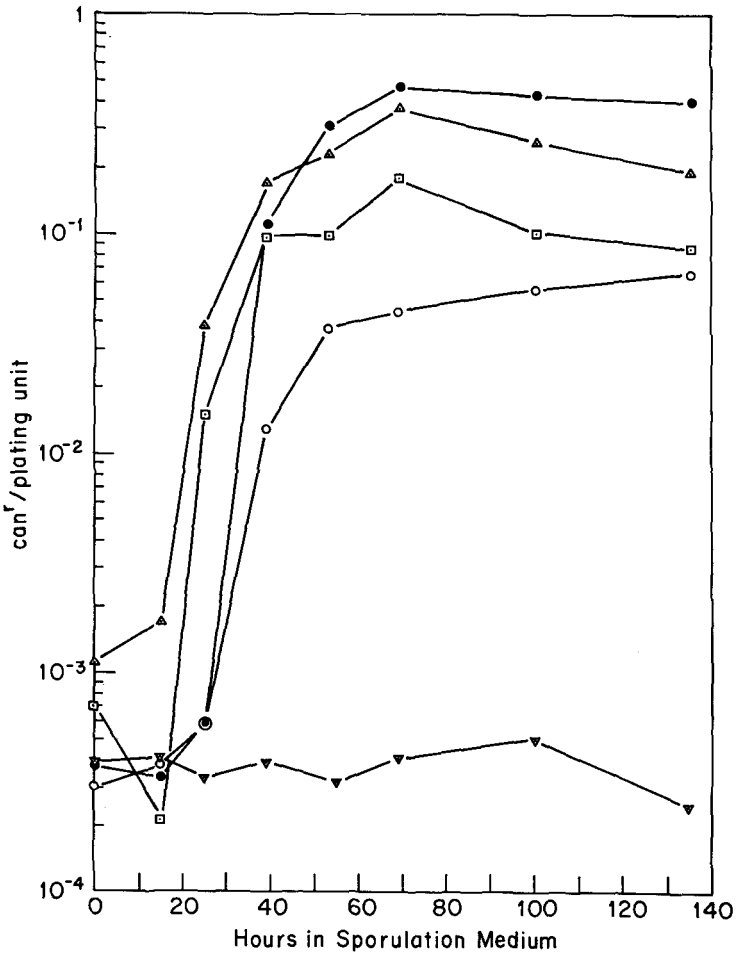

FIGURE 6.—Production of canavanine-resistant cells by incubating in sporulation medium. Cells were pregrown in PSP phthalate, then incubated in KAc, pH7, 3 mM leucine. The frequency of canavanine-resistant cells was determined as described in MATERIALS AND METHODS. ●, +/+; ⊙, *prc1-125/prc1-125*; □, *prb1-9/prb1-1122*; △, *prb1-9 prc1-36/prb1-1122 prc1-36*; ▽, *pep4-3/pep4-3*.

prb1 prc1 and *prc1* diploids also gave substantial frequencies of Can^R colonies (Figure 6 and Table 1). The observation that Can^R colonies occurred with high frequencies in the *prb1*- and/or *prc1*-containing diploids suggested that haploidization was occurring with high frequency, for mitotic recombination would be expected to occur at much lower frequencies, comparable to that seen for the *pep4-3* diploid.

We determined the genotypes of Can^R colonies obtained from these five **a**/α diploids, as well as from **a**/**a** or α/α diploids of selected genotypes. The results are given in Table 1. High frequencies of Can^R clones were obtained from **a**/α diploids of wild-type, *prc1*, *prb1* and *prb1 prc1* genotypes. Low frequencies

TABLE 1

Genetical analysis of canavanine resistant colonies[a]

Relevant genotype	Frequency of *canR* colonies	Number of colonies analyzed	% Haploid	% Meiotic	% Switches
(1) **a** +/α +	0.77[b]	159	71.7[c]	81.1[d]	
(2) **a** +/**a** +	0.9×10^{-4}	160	15.6[e]	33.1[f]	46.9[g]
(3) α +/α +	1.2×10^{-4}	137	13.1[h]	38.0[i]	46.0[j]
(4) **a** *prb1*/α *prb1*	0.69	160	79.4[c]	81.9[d]	
(5) **a** *prc1*/α *prc1*	0.58	160	86.9[c]	88.8[d]	
(6) **a** *prb1 prc1*/α *prb1 prc1*	0.73	160	81.3[c]	86.9[d]	
(7) **a** *pep4–3*/α *per4–3*	2.5×10^{-4}	160	0[c]	0	
(8) **a** *pep4–3*/**a** *pep4–3*	1.3×10^{-4}	160	0	0	1.9[k]

[a] Cells were pregrown in PSP broth and inoculated into KAc, pH 7, 3 mM leucine. Cells were plated at intervals on canavanine-containing medium. Colonies were streaked on YEPD and genotypes determined. These colonies derive from cells plated at 98 hr.
[b] The frequency of Can^R/plating unit at the time of resuspension in sporulation medium ranged from 2.2 to 12×10^{-4} for the eight diploids.
[c] Maters.
[d] Maters plus diploids of other than starting genotype.
[e] α mating type.
[f] Sum of α, any genotype; **a**, not original genotype; nonmater, not original genotype.
[g] Sum of α, any genotype; **a**, not original genotype; nonmater, any genotype.
[h] **a** mating type.
[i] Sum of **a**, any genotype; α, not original genotype; nonmater, not original genotype.
[j] Sum of **a**, any genotype; α, not original genotype; nonmater, any genotype.
[k] Nonmaters.

were seen for the **a**/α diploids bearing *pep4–3* and for the **a**/**a** and α/α diploids. About 70 to 85% of the Can^R colonies obtained from the **a**/α diploids of wild-type and *prb1* and/or *prc1* genotypes were capable of mating and were, therefore, presumably haploid. This conclusion was substantiated by the observation that other recessive markers, also heterozygous in these diploids (*leu1*, *ser1* and, for some diploids, *his5*), were found among the clones. Several of the Can^R clones recovered from the **a**/α diploid homozygous for *prb1* were crossed to a multiply marked haploid. Ten markers on 10 different chromosomes segregated 2:2. Therefore, we concluded that most of the Can^R colonies derived from the *prb1* homozygote, and by extension, the *prb1 prc1* homozygote, were haploid. Some of the remaining colonies were nonmating diploids of a genotype that differed from the initial genotype. These could have arisen from meiototic cells (ESPOSITO and ESPOSITO 1974) or be the result of mating of haploid cells on the plating medium.

The Can^R colonies from the **a**/α diploid homozygous for *pep4–3* aparently arose by mitotic recombination, for all were nonmaters and exhibited the input nutritional requirements. These colonies were not haploid.

The Can^R colonies obtained from the **a**/**a** and α/α wild-type diploids apparently arose by mitotic recombination or by mating-type switch followed by meiosis and sporulation. We infer that the latter process occurred because three classes of Can^R colonies were found that could not be explained by single mitotic recombinational events. These three classes were (1) clones of mating type opposite to that of the parent diploid, (2) nonmating clones and (3) clones with combina-

tions of nutritional markers unlike the parent combination. A low frequency of "switches" was also seen for the *pep4–3* homozygote, although the resulting diploids were not sporulation competent. Whether the frequency of switching is truly lower in the *pep4–3* homozygote cannot be inferred from these data, for the switching event is terminal in the *pep4–3* homozygote, but a prelude to sporulation in the wild-type diploid, and spores would be expected to survive the sporulation conditions better than unsporulated cells.

The genetic data presented in Table 1 clearly indicate that haploidization (and presumably meiosis) was occuring in **a**/α diploids deficient in proteinase B and/or carboxypeptidase Y. Cytological evidence that meiosis was occurring in *prb1* homozygotes is presented in Figure 7. The DNA was stained with DAPI. The apparent "fuzziness" of nuclei of the *prb1* homozygote as compared to nuclei of the wild type was reproducible. To obtain staining of sufficient intensity to permit photography, the *prb1*-bearing meiotic cells must be heated to 37° in the presence of the stain.

In principle, haploid colonies could result from cells blocked in meiosis if only one of the nuclei present in each meiotic cell is viable and gives rise to a colony containing cells of a single genotype. Alternatively, apparent "heterokaryons," resulting from blocked meioses, could be resolved by distribution of meiotic nuclei into separate cells and could give rise to colonies containing cells of several genotypes. To determine whether only one nucleus per meiotic cell was surviving or whether segregation from the apparent heterokaryon was occurring, we allowed asci (wild type) or cells with carved out cytoplasm (*prb1*) to grow on YEPD medium for 15 hr and then streaked the microcolonies on YEPD. The nutritional requirements and mating type of a number of clones derived from each microcolony were determined. From these phenotypes we could infer the minimum number of nuclei contributing to daughter clones. (Where haploids and diploids, or more than one diploid genotype, were present, we could infer the minimum number of parents for the genotypes present.) The data are presented in Table 2. It is clear that more than one nucleus contributes to the progeny of the *prb1* "sporulative" cells. One of 21 asci from the wild type showed an abnormal marker distribution; whereas, 4 of 41 cells from the *prb1* mutant showed an abnormal

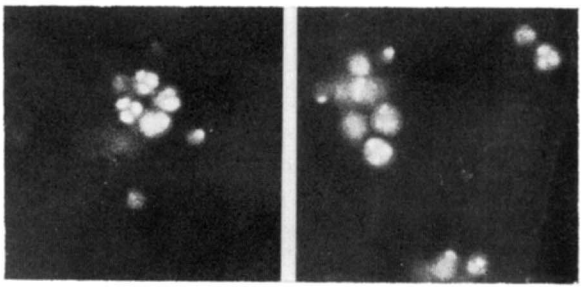

FIGURE 7.—Staining of meiotic nuclei with DAPI after exposure of **a**/α diploids to sporulation regimen after pregrowth on PSP broth. Staining was performed as described in MATERIALS AND METHODS. +/+ homozygote is on the left; *prb1–9/prb1–1122* diploid is on the right (× 400).

distribution (*e.g.*, three different genotypes among segregants of **a** mating type). The results of this series of genetic and cytological experiments indicate that the *prb1* homozygotes undergo meiosis. Whether heterokaryons or spores were produced was not discernible from the data. Visual examination of glusulase-treated *prb1* "sporulative" cells revealed no spores.

In order to determine whether spores or heterokaryons were present, we treated sporulative cells of wild-type, *prb1* and *pep4–3* genotypes, all of which were heterozygous for *can1*, with glusulase and dithiothreitol, followed by sonication. This treatment should be lethal to heterokaryons, but have little or no effect on the viability of spores, and should dissociate the spores. The results are given in Table 3. Both pregrowth conditions employed in previous experiments were tested.

The frequency of Can^R cells in the population, shown in Column 3, should reflect the frequency of meiosis (except for the *pep4–3* homozygote, where it reflects the frequency of mitotic recombination). Columns 4 and 5 provide the number of plating units present in the cultures before and after treatment. Column 6 is the ratio of the number of plating units present following treatment to that present before treatment. Two expected ratios were calculated and are shown in column 7. The first (b) assumes that each dissociated ascus will give four plating units and that vegetative cells live; the second (c) assumes that vegetative cells die. Columns 8 and 9 are the numbers of Can^R plating units before and after treatment, column 10 is the ratio of the two. The expected ratio, column 11, is two, because each ascus should give two Can^R spores after treatment.

Consider the results for the *pep4–3* diploid. These cells do not sporulate. All should be killed by the treatment. The expected ratio of viable cells after treatment to before treatment would be one if vegetative cells were not killed, and zero if they were (the expectation 5.6×10^{-5}, line 5, calculated by the metric, assumes that Can^R cells derive from meiosis, which they do not). It is clear that the treatment killed vegetative cells of the *pep4–3* homozygote, for the ratio was nearly zero (3.6×10^{-4}).

For the wild-type diploid, the expected ratio of plating units in the culture following glusulase tretament to plating units before glusulase treatment is 3.55 if vegetative cells are not killed (line 2) and 3.40 if they are. The number of plating

TABLE 2

Minimum number of haploid nuclei contributing to the progeny of a sporulative cell or ascus

Inferred no. of haploid nuclei	No. of cells with inferred no. of haploid nuclei	
	+/+	*prb1/prb1*
1	0	5
2	11	20
3	7	14
4	3	2
Total examined	21	41

TABLE 3

Sensitivity of meiotic products to glusulase-dithiothreitol[a]

	Relevant genotype	Pregrowth medium	Frequency Can^R	Viable cells/ml -glusulase $\times 10^6$	Viable cells/ml +glusulase $\times 10^6$	Viable cells/ml +glusulase/-glusulase Observed	Viable cells/ml +glusulase/-glusulase Expected	Can^R cells/ml -glusulase $\times 10^6$	Can^R cells/ml +glusulase $\times 10^6$	Can^R cells/ml +glusulase/-glusulase Observed	Can^R cells/ml +glusulase/-glusulase Expected
(1)	+/+	PSP phthalate	0.80	8.08	14.1	1.75	3.40[b] 3.20[c]	6.45	7.15	1.11	2
(2)	+/+	PSP broth	0.85	12.4	23.4	1.89	3.55[b] 3.40[c]	10.6	14.1	1.33	2
(3)	prb1-9 / prb1-1122	PSP phthalate	0.13	3.40	0.51	0.15	1.39[b] 0.52[c]	0.43	0.24	0.56	2
(4)	prb1-9 / prb1-1122	PSP broth	0.26	6.45	2.06	0.32	1.78[b] 1.04[c]	1.49	1.13	0.76	2
(5)	pep4-3 / pep4-3	PSP phthalate	1.4×10^{-5}	3.68	0.001	2.64×10^{-4}	1[b] 5.6×10^{-5}[c]	0.00005	0.00003	0.6	2
(6)	pep4-3 / pep4-3	PSP broth	1.3×10^{-4}	14.6	0.052	3.6×10^{-4}	1[b] 5.2×10^{-4}[c]	0.002	0	0	2

[a] Cells were pregrown in PSP phthalate or PSP broth and incubated in KAc, pH 7, 3 mM leucine. After 95 hr of incubation, they were plated with or without glusulase-dithiothreitol treatment.

[b] $4 \times (\text{frequency of } Can^R)_{-glus} + (1 - \text{frequency of } Can^R)_{-glus}$

[c] $4 \times (\text{frequency of } Can^R)_{-glus}$.

units after treatment is increased, but only 1.9-fold. The expected ratio assumes perfect dissociation of spores and that all asci will have four spores, although neither assumption is correct. We attribute the deviation from expectation to this fact. The expected ratio of Can^R plating units following treatment to Can^R plating units before treatment is two. The observed ratio is greater than one (1.33), but not nearly two. We infer that incomplete dissociation and the presence of asci bearing fewer than four spores lowered this ratio as well.

A similar evaluation of the data for the *prb1* homozygote leads to expected ratios of viable cells of 0.52 if pregrowth is on PSP phthalate and 1.04 if pregrowth is on PSP broth, assuming that vegetative cells are killed. The observed ratios are substantially below this expectation Indeed, the ratios of observed/expected for the wild type in the two pregrowth conditions are 0.55 and 0.56. For the *prb1* diploids, comparable ratios are 0.29 and 0.31.

If the Can^R colonies from *prb1* homozygotes derive from mature spores, the expected ratio of the number of Can^R plating units present following glusulase DTT treatment to that present before treatment is two, just as it was for wild type. Contrary to what was seen for wild type, however, the observed ratio is less than one. Therefore, a substantial fraction of the meiotic products of the *prb1* homozygote are killed by the glusulase-DTT treatment. The ratios of observed to expected for the wild type in the two pregrowth conditions are 0.56 and 0.67. Comparable number for *prb1* are 0.28 and 0.38.

It is difficult to draw firm conclusions from these experiments, for technical difficulties abound. We have no measure of the extent of dissociation, nor of the distribution of 2-, 3- and 4-spored asci. We could obtain the latter for the wild-type diploid, but not for the *prb1* homozygote. Some conclusions are allowable, however.

The "sporulative" cells produced from the *prb1* homozygote are vastly more resistant to glusulase-DTT treatment than are the nonsporulating cells of *pep4–3* genotype. The *prb1* "sporulative" cells thus are not heterokaryons. On the other hand, "spores" derived from *prb1* cells are less resistant to glusulase dithiothreitol treatment than are wild-type spores.

We inferred from the above experiments that nearly normal spores were being produced in *prb1* homozygotes. For this reason, we attempted to dissect spores from "sporulative" cells of *prb1* homozygotes after routine glusulase (no DTT) treatment. This involved identifying a suitably sized lump of debris that bore some resemblance to a "sporulative" cell. We were able to tease out spores from such debris. Three of the four spores were very small, but normal in appearance. The fourth was not classifiable visually as a spore, for it was tiny and did not have the refractile appearance of a normal spore. Nearly all of these "spores" germinated and produced colonies.

DISCUSSION

From the results that we have presented, we conclude that the protein degradation that occurs during sporulation is not specific, but may be essential if sporulation is to proceed to completion. Cells of all proteinase genotypes (except *pep4–3*

homozygotes) undergo meiosis and form spores, albeit small and possibly abnormal ones in the case of the *prb1* homozygotes. About 40–50% of the protein degradation observed is catalyzed by proteinase B and carboxypeptidase Y in combination. Neither carboxypeptidase Y nor proteinase B is required for spore germination.

In the experiments reported here, at least 50% of the vegetative proteins were degraded during sporulation of wild-type yeast cells, measured as loss of acid-precipitable radioactivity from prelabelled cells. This value is somewhat lower than that (60–70%) reported by BETZ and WEISER (1976), but substantially higher than that (30%) reported by KLAR and HALVORSON (1975). At least part of the discrepancy between our results and those of BETZ and WEISER (1976) may arise from strain differences, for their strain sporulates about 1.5 times more rapidly than do our strains. Possibly the lower value seen by KLAR and HALVORSON (1975) arises from their use of labelled arginine.

When release of radioactive methionine into the medium was used as a measure of degradation. HOPPER *et al.* (1974) found that only 25–30% of the label was released. We found 50% release, using identical procedures (compared to 75% for leucine). Again major strain differences are present, for our strains take twice as long to sporulate as theirs.

The protein degradation seen in our experiments is not sporulation specific, for nearly identical patterns and extents of degradation are seen for near isogenic **a**/**a** and **a**/α diploids of all five proteinase genotypes. This is in marked contrast to results reported by HOPPER *et al.* (1974), but is basically in agreement with results reported for a haploid of **a** mating type by BETZ and WEISER (1976). It seems likely that the protein degradation is a response to nitrogen starvation, rather than part of process of sporulation *per se*. The rates of degradation observed in our experiments (2–3%/hr) are similar to the 2.5–3%/hr reported by BETZ and WEISER (1976). Both rates are quite comparable to the 2%/hr observed when vegetative cells are starved of nitrogen (LOPEZ and GANCEDO 1979).

One cannot conclude from the data that we have presented that extensive protein degradation is required if sporulation is to proceed normally, even though there are strong correlations between sporulation competence and high levels of protein degradation. However, it seems unlikely that the only requirement for proteinase B activity during sporulation is for degradation of one or a few specific proteins, for sporulation proceeds nearly to completion in a *prb1* homozygote. For these and other reasons, we consider it likely that some level of nonspecific protein degradation is required during sporulation.

Because the process of proteolysis may be crucially important to supply the amino acids necessary for synthesis of new proteins during sporulation, the addition of amino acids to cultures of proteinase deficient cells that have become committed to sporulation (ESPOSITO and ESPOSITO 1974) might have been expected to allow these cells to complete sporulation. We attempted these experiments using buffered sporulation media that preserve the ability of cells to take up amino acids (MCCUSKER and HABER 1977). No improvement in ascus for-

mation was observed for any strain. Indeed, the use of these media for our strains resulted in reduced frequencies of meiosis and sporulation for all strains, including wild type.

The proteinase-deficient mutants show reduced rates and extents of protein degradation. The result is most marked for the *pep4–3* homozygote (60–75% reduction), a result difficult to interpret because of the pleiotropic effects of the mutation (JONES 1977; HEMMINGS, ZUBENKO and JONES 1980; JONES *et al.* 1980). Deficiency for carboxypeptidase Y decreases degradation by 10–20%; deficiency for proteinase B decreases degradation by 40–50%. The proteinase B deficiency is epistatic to the carboxypeptidase Y deficiency, implying that proteinase B provides the substrates upon which carboxypeptidase Y acts, despite the presence in these cells of the endoproteinase proteinase A. Whether the *prc1*-bearing mutant accumulates undegraded oligopeptides is an unexplored question.

The frequency of meiosis in proteinase-deficient strains is usually lower than that of the wild type (Figure 6, Tables 1 and 3). This difference in frequency is not responsible for the deficit in protein degradation in the mutants because (1) the doubly mutant diploid *prb1 prc1* had a high frequency of meiosis in some experiments (Figure 6), yet protein degradation was 50% of wild type, and (2) asporogenous **a**/**a** diploids showed rates of degradation comparable to those of the **a**/α diploids. One can infer from the experiments on protein degradation that proteinase B and carboxypeptidase Y are involved in this degradation. Indeed, together they account for 40–50% of the degradation.

It is clear that all of the mutant **a**/α diploids except the *pep4–3* homozygote can undergo meiosis. However, *prb1* homozygotes do not exhibit normal sporulation. Spores are formed, but the asci are clearly abnormal. The spores, smaller than usual and sometimes tiny, remain embedded in a thick matrix. Whether the spores are completely normal is difficult to assess. Clearly some of them are killed by glusulase-DTT treatment, for the absolute number of viable Can^R cells drops upon glusulase treatment. There are two possible explanations of this observation, however. (1) Spores from the *prb1* homozygote are abnormal and are more sensitive to glusulase-DTT treatment than are wild-type spores, or (2) spores from the wild type and the *prb1* homozygote are equally sensitive to glusulase-DTT treatment. One detects killing of spores of the *prb1* homozygote because the matrix in which the spores are embedded prevents dissociation; whereas, the killing of wild-type spores remains undetected because the treatment dissociates the spores, the number of plating units rises and the final ratio reflects the balance between dissociation and killing. We are unable to distinguish between these possibilities.

Carboxypeptidase Y is not required for spore germination, since spores from the *prc1* homozygote germinate. Proteinase B would appear not to be required for spore germination, for spores from the *prb1* homozygote can germinate. Whether this enzyme is required for germination of completely normal spores must remain an open question.

Mutations in two previously described genes, *KEX2* and *GCN1*, render sporulation abnormal, but do not block meiosis. The *kex2* mutants undergo meiosis without producing visible spores, are defective in expression and secretion of

killer toxin, α-factor and other secreted proteins, and render the α genotype sterile (LEIBOWITZ and WICKNER 1976; ROGERS, SAVILLE and BUSSEY 1979). The *prb1* mutants are clearly different from *kex2* mutants, for *prb1* mutants support killer and express killer toxin (ZUBENKO, MITCHELL and JONES 1980), mate normally in either mating type and produce normal α-factor halos (unpublished observations).

The glucosamine-requiring mutants bearing *gcn1* undergo meiosis and produce spores, but the spores are not refractile, are sensitive to glusulase and lack the outer layer of the spore wall. Their glusulase sensitivity is much more marked than that of the spores produced from *prb1* homozygotes (WHELAN and BALLOU 1975; BALLOU *et al.* 1977).

Vacuoles have been reported to fragment and eventually disappear during sporulation (SVIHLA, DAINKO and SCHENK 1964; MARQUARDT 1963). It is clear from the electron micrographs presented by SCHWENKE, MAGANA-SCHWENKE and LAPORTE (1977) that vacuoles are present in mature asci and, indeed, fill nearly the entirety of the cytosol around the spores. This location corresponds to that of the matrix material that remains in the "sporulated" cells of the *prb1* homozygote. We do not yet know what happens to the vacuole during sporulation in the *prb1* mutant. A question that arises, regardless of the dynamics of the vacuole, is whether the protein degradation that occurs during sporulation occurs within the vacuole where the proteinases are located during vegetative growth (see WIEMKE, SCHELLENBERG and URECH 1979 for a summary of vacuolar enzymes), which would require transport of the proteins to be degraded into the vacuole, or rather involves the discharge of the proteinases into the cytosol. Regardless of which mechanism is operative, dissolution of the matrix material, in which spores of *prb1* diploids remain embedded, requires proteinase B activity. It is tempting to speculate that dissolution of the matrix material and hence proteinase B activity is an essential part of the normal spore-dispersal mechanism in nature.

We report here that *prb1* homozygotes undergo meiosis, produce spores, albeit small and possibly abnormal ones, but do not produce asci of normal appearance. WOLF and EHMAN (1970 reported that *prb*⁻ homozygotes form asci, but at half the rate and to half the extent of wild-type cells. It is difficult to evaluate the basis of this discrepancy. We do not know what the basis of the proteinase B defect is in their mutant, whether the mutation lies in the structural gene, and, if so, what the nature of the mutation is. The diploids compared were not isogenic; hence, differences in rates or extents of ascus formation are uninterpretable.

We thank BARBARA MITCHELL and SARAH MALLICK for skilled technical assistance and JOHN TALIAN and BONNIE CHOJNACKI for the photomicrographs. This work was supported by Public Health Service grant AM18090 to ELIZABETH W. JONES.

LITERATURE CITED

BALLOU, C., S. MAITRA, J. WALKER and W. WHELAN, 1977 Developmental defects associated with glucosamine auxotrophy in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U.S. **74**: 4351–4355.

BETZ, H., 1975 Protein degradation and proteinase during yeast sporulation. pp. 73. In: *The Molecular Biology of Yeast.* Edited by D. BOTSTEIN and G. FINK. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. ——, 1979 Loss of sporulation ability in a yeast mutant with low proteinase A levels. FEBS Letters **100:** 171–174.

BETZ, H. and U. WEISER, 1976 Protein degradation and proteinases during yeast sporulation. Eur. J. Biochem. **62:** 65–72.

CABIB, E., R. ULANE and B. BOWERS, 1973 Yeast chitin synthetase: separation of the zymogen from its activating factor and recovery of the latter in the vacuole fraction. J. Biol. Chem. **248:** 1451–1458.

CHEN, A. and J. MILLER, 1968 Proteolytic activity of intact yeast cells during sporulation. Can. J. Microbiol. **14:** 957–963.

ESPOSITO, M., R. ESPOSITO, M. ARNAUD and H. HALVORSON, 1969 Acetate utilization and macromolecular synthesis during sporulation of yeast. J. Bacteriol. **100:** 180–186.

ESPOSITO, R. and M. ESPOSITO, 1974 Genetic recombination and commitment to meiosis in *Saccharomyces.* Proc. Natl. Acad. Sci. U.S. **71:** 3172–3176.

HASILIK, A., H. MÜLLER and H. HOLZER, 1974 Compartmentation of the tryptophan-synthase-proteolyzing system in *Saccharomyces cerevisiae.* Eur. J. Biochem. **48:** 111–117.

HAYASHI, R., S. AIBARA and T. HATA, 1970 A unique carboxypeptidase activity of yeast proteinase C. Biochim. Biophys. Acta **212:** 359–361.

HAYASHI, R., S. MOORE and W. STEIN, 1973 Carboxypeptidase from yeast. Large scale preparation and the application to COOH-terminal analysis of peptides and proteins. J. Biol. Chem. **248:** 2296–2302.

HEMMINGS, B., G. ZUBENKO and E. JONES, 1980 Proteolytic inactivation of the NADP-dependent glutamate dehydrogenase in proteinase deficient mutants of *Saccharomyces cerevisiae.* Arch. Biochem. Biophys. **202:** 657–660.

HOPPER, A., P. MAGEE, S. WELCH and B. HALL, 1974 Macromolecule synthesis and breakdown in relation to sporulation and meiosis in yeast. J. Bacteriol. **119:** 619–628.

JONES, E., 1977 Proteinase mutants of *Saccharomyces cerevisiae.* Genetics **85:** 23–33.

JONES, E. and K. LAM, 1973 Mutations affecting levels of tetrahydrofolate interconversion enzymes in *Saccharomyces cerevisiae.* II. Map positions on chromosome VII of *ade3–41* and *ADE15.* Molec. Gen. Genet. **123:** 209–218.

JONES, E., H. LUND and G. ZUBENKO, 1975 Proteinase and peptidase mutants of *S. cerevisiae.* p. 74. In: *The Molecular Biology of Yeast.* Edited by D. BOTSTEIN and G. FINK. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

JONES, E., G. ZUBENKO, R. PARKER, B. HEMMINGS and A. HASILIK, 1980 Pleiotropic mutations of *S. cerevisiae* which cause deficiency for proteinases and other vacuole enzymes. In: Alfred Benzon Symposium 16, *Molecular Genetics in Yeast.* Edited by D. VON WETTSTEIN, J. FRIIS, M. KIELLAND-BRANDT and A. STENDERUP. Munksgaard, Copenhagen (in press).

KLAR, A. and H. HALVORSON, 1975 Proteinase activities of *Saccharomyces cerevisiae* during sporulation. J. Bacteriol. **124:** 863–869.

LEIBOWITZ, M. and R. WICKNER, 1976 A chromosomal gene required for killer plasmid expression, mating and spore maturation in *Saccharomyces cerevisiae.* Proc. Natl. Acad. Sci. U.S. **73:** 2061–2063.

LENNEY, J., P. MATILE, A. WIEMKEN, M. SCHELLENBERG and J. MEYER, 1974 Activities and cellular localization of yeast proteases and their inhibitors. Biochem. Biophys. Res. Comm. **60:** 1378–1383.

LOPEZ, S. and J. GANCEDO, 1979 Effect of metabolic condition on protein turnover in yeast. Biochem. J. **178:** 769–776.

MARQUARDT, H., 1963 Elektronenoptische untersuchungen über die ascosporenbildung bei *Saccharomyces cerevisiae* unter cytologischem und cytogenetischem aspekt. Arch. Mikrobiol. **46:** 308–320.

MATERN, H., H. BETZ and H. HOLZER, 1974 Compartmentation of inhibitors of proteinases A and B and carboxypeptidase Y in yeast. Biochem. Biophys. Res. Comm. **60:** 1051–1059.

MCCUSKER, J. and J. HABER, 1977 Efficient sporulation of yeast in media buffered near pH 6. J. Bacteriol. **132:** 180–185.

MITCHELL, A. and E. JONES, 1977 Protease B mutants of *Saccharomyces cerevisiae*. p. 35. In: *The Molecular Biology of Yeast*. Edited by F. SHERMAN and J. BROACH. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

ROGERS, D., D. SAVILLE and H. BUSSEY, 1979 *Saccharomyces cerevisiae* killer expression mutant *kex2* has altered secretory protein and glycoproteins. Biochem. Biophys. Res. Comm. **90:** 187–193.

ROTH, R. and H. HALVORSON, 1969 Sporulation of yeast harvested during logarithmic growth. J. Bacteriol. **98:** 831–832.

SCHWENKE, J., N. MAGANA-SCHWENKE and J. LAPORTE, 1977 Yeast protoplasts from stationary and starved cells: preparation, ultrastructure and vacuolar development. Annales de Microbiologie **128A:** 3–18.

SVIHLA, G., J. DAINKO and F. SCHENK, 1964 Ultraviolet microscopy of the vacuole of *Saccharomyces cerevisiae* during sporulation. J. Bacteriol. **88:** 449–456.

WHELAN, W. and C. BALLOU, 1975 Sporulation in D-glucosamine auxotrophs of *Saccharomyces cerevisiae*: Meiosis with defective ascospore wall formation. J. Bacteriol. **124:** 1545–1557.

WIEMKEN, A., M. SCHELLENBERG and K. URECH, 1979 Vacuoles: the sole compartments of digestive enzymes in yeast (*Saccharomyces cerevisiae*)? Arch. Microbiol. **123:** 23–35.

WILLIAMSON. D. and D. FENNELL, 1975 The use of fluorescent DNA-binding agent for detecting and separating yeast mitochondrial DNA. pp. 335–351. In: *Methods in Cell Biology XII*. Edited by D. PRESCOTT. Academic Press, New York.

WOLF, D., I. BECK and C. EHMAN, 1979 The intracellular proteinases and their inhibitors in yeast: a genetic and biochemical approach to their function. pp. 61–64. In: *Limited Proteolysis in Microorganisms*. Edited by H. HOLZER and G. COHEN. DHEW Publ. No. 79–1591.

WOLF, D. and C. EHMAN, 1978 Isolation of yeast mutants lacking proteinase B activity. FEBS Letters **92:** 121–124.

WOLF, D. and C. EHMAN, 1979 Studies on a proteinase B mutant of yeast. Eur. J. Biochem. **98:** 375–384.

WOLF, D. and G. FINK, 1975 Proteinase C (carboxypeptidase Y) mutant of yeast. J. Bacteriol. **123:** 1150–1156.

WOLF, D. and U. WEISER, 1977 Studies on a carboxypeptidase Y mutant of yeast and evidence for a second carboxypeptidase activity. Eur. J. Biochem. **73:** 553–556.

ZUBENKO, G., A. MITCHELL and E. JONES, 1978 Identification of the structural gene for protease B in *S. cerevisiae*. p. 99. In: 9th *International Conference on Yeast Genetics and Molecular Biology*. University of Rochester, Rochester, N.Y. ——, 1979a Cell division, catabolite inactivation, and sporulation in protease B deficient mutants of *Saccharomyces cerevisiae*. pp. 49–53. In: *Limited Proteolysis in Microorganisms*. Edited by H. HOLZER and G. COHEN. DHEW Publication No. 79–1591. ——, 1979b Septum formation, cell division and sporulation in mutants of yeast deficient in proteinase B. Proc. Natl. Acad. Sci. U.S. **76:** 2395–2399. ——, 1980 Mapping of the proteinase B structural gene, *PRB1*, in *Saccharomyces cerevisiae* and identification of nonsense alleles within the locus. Genetics **96:** 137–146.

Corresponding editor: D. BOTSTEIN