

# Autophagy in Yeast Demonstrated with Proteinase-deficient Mutants and Conditions for its Induction

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**Abstract.** For determination of the physiological role and mechanism of vacuolar proteolysis in the yeast *Saccharomyces cerevisiae*, mutant cells lacking proteinase A, B, and carboxypeptidase Y were transferred from a nutrient medium to a synthetic medium devoid of various nutrients and morphological changes of their vacuoles were investigated. After incubation for 1 h in nutrient-deficient media, a few spherical bodies appeared in the vacuoles and moved actively by Brownian movement. These bodies gradually increased in number and after 3 h they filled the vacuoles almost completely. During their accumulation, the volume of the vacuolar compartment also increased. Electron microscopic examination showed that these bodies were surrounded by a unit membrane which appeared thinner than any other intracellular membrane. The contents of the bodies were morphologically indistinguishable from the cytosol; these bodies contained cytoplasmic ribosomes, RER, mitochondria, lipid granules and glycogen granules, and the density of the cytoplasmic ribosomes in the bodies was almost the same as that of ribosomes in the cytosol. The diameter of the bodies ranged from 400 to 900 nm. Vacuoles that had accumulated these bodies were prepared by a modification of the method of Ohsumi and Anraku (Ohsumi, Y., and Y. Anraku. 1981. *J. Biol.*

*Chem.* 256:2079–2082). The isolated vacuoles contained ribosomes and showed latent activity of the cytosolic enzyme glucose-6-phosphate dehydrogenase. These results suggest that these bodies sequestered the cytosol in the vacuoles. We named these spherical bodies “autophagic bodies.” Accumulation of autophagic bodies in the vacuoles was induced not only by nitrogen starvation, but also by depletion of nutrients such as carbon and single amino acids that caused cessation of the cell cycle.

Genetic analysis revealed that the accumulation of autophagic bodies in the vacuoles was the result of lack of the *PRB1* product proteinase B, and disruption of the *PRB1* gene confirmed this result. In the presence of PMSF, wild-type cells accumulated autophagic bodies in the vacuoles under nutrient-deficient conditions in the same manner as did multiple protease-deficient mutants or cells with a disrupted *PRB1* gene. As the autophagic bodies disappeared rapidly after removal of PMSF from cultures of normal cells, they must be an intermediate in the normal autophagic process.

This is the first report that nutrient-deficient conditions induce extensive autophagic degradation of cytosolic components in the vacuoles of yeast cells.

**M**OST eukaryotic cells are known to have specific intracellular systems for degrading their own obsolete proteins or organelles. In animal cells, both lysosomal and nonlysosomal pathways are responsible for the turnover of endogenous proteins. Many types of nonlysosomal protein degradation pathways have been reported (Pontremoli and Melloni, 1986); namely, signal peptidases, Ca<sup>2+</sup>-activated neutral proteinases, and the ubiquitin-mediated ATP-dependent protein degradation pathway (Schlesinger and Hershko 1988; Rechsteiner, 1991). These pathways are specific for target substrates and are mainly responsible for the degradation of short-lived or abnormal proteins and for the activation of immature proteins.

Lysosomal protein degradation is performed by autophago-

lysosomes which are formed by fusion of autophagosomes and vesicles containing lysosomal proteases or lysosomal membrane proteins. Autophagosomes are usually formed to degrade excess cellular components whose roles are finished or to degrade parts of cellular components for supplying required nutrients. Formation of autophagosomes in rat liver on depletion of amino acids has been reported (Schworer and Mortimore, 1979). Protein degradation by autophagy is thought to be responsible for bulk turnover of proteins. Autophagy can result in drastic protein degradation at a rate of 3–4% of the total cellular protein per hour and this degradation is thought to be nonselective, at least in the case of cytosolic enzymes (Kopitz et al., 1990 and references therein).

In plant cells, degradation processes are sometimes even

more drastic. Most proteins in senescent leaves of higher plants are degraded, and the degradation products are transported to storage compartments through sieve tubes. Vacuoles, containing most of the cellular proteases, are thought to be functionally equivalent to animal lysosomes and to be responsible for the bulk turnover of proteins. Matile (1975) showed that the activities of vacuolar proteases increase during senescence of leaves and fading of flowers. This strongly suggested the role of vacuoles in cellular protein degradation, but did not provide unequivocal evidence of degradation of intrinsic proteins in the vacuoles.

Cells of *Saccharomyces cerevisiae* usually contain a few vacuoles. Major proteases, such as proteinase A and B, carboxypeptidase Y, and amino peptidase I, are localized in these vacuoles (Wiemken et al., 1979). These enzymes and their biogenesis have been studied extensively (Achstetter and Wolf, 1985; Klionsky et al., 1988, 1990). Several specific roles of these proteinases have been proposed. Recently, Chiang and Schekman (1991) suggested that the vacuolar proteinases may be involved in the degradation of the key regulatory enzyme in gluconeogenesis, fructose 1,6-bisphosphatase. The levels of these vacuolar proteases vary with the carbon and nitrogen sources supplied to the cells (Distel et al., 1983; Hansen et al., 1977; Saheki and Holzer, 1975). They also change with the growth stage, reaching maximum levels when the cells approach the stationary phase (Frey and Röhm, 1978; Trumbly and Bradley, 1983). There are reports that drastic degradation of cellular proteins occurs (Esposito et al., 1969; Hopper et al., 1974) and the levels of several vacuolar proteases increase during sporulation (Betz and Weiser, 1976; Klar and Halvorson, 1975; Chen and Miller, 1968). Indeed, cells lacking proteinase A and B activities could not undergo the normal sporulation process (Mechler and Wolf, 1981; Teichert et al., 1989; Wolf and Ehmann, 1979; Zubenko and Jones, 1981). These findings suggest that proteolysis in the vacuoles of yeast is essential in protein turnover in nutrient-deficient conditions. However, the detailed mechanism of protein degradation in the vacuoles and its contribution to the total protein turnover are still unclear. In this work, using strains lacking vacuolar proteases, we demonstrated for the first time that active autophagy occurs in yeast in various adverse conditions.

## Materials and Methods

### Strains and Growth Conditions

The strains of *S. cerevisiae* used in this work are listed in Table I. Cells were cultured until the middle or end of the logarithmic growth phase at 30°C on a rotary shaker in nutrient medium YEPD consisting of 1% yeast extract (Difco Laboratories, Inc., Detroit, MI), 2% polypepton (Wako, Osaka, Japan), and 2% glucose, or in a synthetic complete medium, SD (see below) supplemented with 0.2 g/l of casamino acids (Difco Laboratories, Inc.), and 20 mg/l each of adenine sulfate and uracil. The cells were harvested by centrifugation (3,000 rpm × 3 min), washed once with distilled water, resuspended in a synthetic medium at a density of  $2 \times 10^7$  cells/ml, and incubated at 30°C on a rotary shaker. The synthetic media used were SG and SD: 6.7 g/l of yeast nitrogen base (Difco Laboratories, Inc.) with 2% glycerol and 2% glucose, respectively; SG(-N) and SD(-N): 1.7 g/l of yeast nitrogen base without amino acids and ammonium sulfate (Difco Laboratories, Inc.), with 2% glycerol and 2% glucose, respectively; SG(-A) and SD(-A): 6.7 g/l yeast nitrogen base without amino acids (Difco Laboratories, Inc.), with 2% glycerol and glucose, respectively. When indicated, the following nutrients were added: adenine sulfate or uracil, 20 mg/l; L-leucine, 60 mg/l and L-tryptophan, 20 mg/l. Synthetic medium without sulfate

was prepared by replacing all the sulfate salts by chloride salts. Cells cultured in YEPD medium and in the synthetic complete medium described above gave essentially the same results when transferred to a starvation medium. For convenience, as a standard experimental procedure, cells grown in YEPD were used. The strains originally constructed by E. W. Jones (1984) could not use glycerol as a carbon source, so in SG medium they were exposed to carbon deficiency. The  $\rho^0$  strain was constructed as described by Sherman et al. (1974).

### Vacuole Isolation

Yeast vacuoles were isolated by the procedure of Ohsumi and Anraku (1981) with slight modifications. Briefly, cells were grown in YEPD to a density of  $4\text{--}5 \times 10^7$  cells/ml. Then they were harvested, washed once with distilled water, and incubated for 1.5 to 4.5 h in SG medium supplemented with the required nutrients. Cells were converted to spheroplasts by incubation for 40–50 min in medium containing 50 mM Tris-HCl (pH 7.5), 1 M sorbitol, 40 mM mercaptoethanol, 10 mM Na<sub>3</sub>N, and 3 U/ml of zymolyase 20T at a density of  $8\text{--}10 \times 10^7$  cells/ml. The spheroplasts were collected and washed twice with medium containing 50 mM Tris-HCl (pH 7.5), 1 M sorbitol, and 10 mM Na<sub>3</sub>N, and were ruptured in buffer A (10 mM MES-Tris, pH 6.9, 0.1 mM MgCl<sub>2</sub>, and 12% (wt/vol) Ficoll 400) in a Dounce homogenizer at 4°C. The lysate was transferred to centrifuge tubes and an equal volume of buffer B (10 mM MES-Tris, pH 7.5, 0.5 mM MgCl<sub>2</sub>, and 8% wt/vol Ficoll 400) was layered on top, and centrifuged at 33,000 g for 30 min. The white layer at the top of the tube was collected, and resuspended in buffer B. This crude vacuole suspension was transferred to centrifuge tubes and a step-wise gradient of 4% and 0% Ficoll 400 in buffer B was layered on top, and centrifuged at 33,000 g for 30 min, and vacuoles were collected from the interface of 0% and 4% Ficoll 400. Control vacuoles were prepared from cultures at 0 h of starvation.

### Enzyme Assay

Glucose-6-phosphate dehydrogenase (G6PDH)<sup>1</sup> activity was measured by the method of Löhr and Waller (1974). Its latent activity was measured after two cycles of freezing–thawing or sonication of samples.

### Protein Assay

Protein concentration was assayed by the method of Lowry et al. (1951) with BSA as standard.

### Light and Fluorescence Microscopies

Cells were examined under an Olympus BH2 microscope (Tokyo, Japan) with a 100× oil-immersion objective, equipped for fluorescence or phase contrast optics, or a Zeiss Axioplan microscope (Oberkochen, Germany), equipped for Nomarsky optics. Images were photographed with T-MAX ASA400 film with a T-MAX developer (Eastman Kodak Co., Rochester, NY).

### Vital Staining

4',6-Diamidino-2-phenylindole (DAPI, 1 μg/ml) was added to growing cells in nutrient-deficient medium 1 h before their observation. DAPI fluorescence was examined using UV-excitatory illumination.

Acidification of vacuoles was estimated with quinacrine as follows. The pH of logarithmic cultures in YEPD was adjusted to pH 7.5 with 50 mM phosphate buffer and then quinacrine was added at a final concentration of 100 μM. The cells were incubated for 5 min at 30°C on a rotary shaker, washed with 50 mM phosphate buffer (pH 7.5) by centrifugation, resuspended in a small volume of phosphate buffer, and examined with a fluorescence microscope.

### Electron Microscopy

Harvested cells were promptly frozen in melting Freon 22 cooled with liquid nitrogen, as described in a previous paper (Baba and Osumi, 1987). Ultrathin sections were examined with a Hitachi H-500H electron microscope at 100 kV.

1. *Abbreviations used in this paper:* DAPI, 4',6-diamidino-2-phenylindole; G6PDH, glucose-6-phosphate dehydrogenase; PrA, proteinase A; PrB, proteinase B.

Table I. Yeast Strains Used

Strains	Genotype	Source or reference
X2180-1B	<i>MAT<math>\alpha</math> SUC2 mal mel gal2 CUP1</i>	Y.G.S.C.
BJ926	<i>MAT<math>\alpha</math> prb1-1122 prc1-407 pep4-3 can1 gal2 his +</i> <i>MAT<math>\alpha</math> prb1-1122 prc1-407 pep4-3 can1 gal2 + trp</i>	Y.G.S.C.
BJ1991	<i>MAT<math>\alpha</math> prb1-1122 pep4-3 leu2 trp1 ura3-52</i>	Y.G.S.C.
BJ2168	<i>MAT<math>\alpha</math> prb1-1122 prc1-407 pep4-3 leu2 trp1 ura3-52</i>	Y.G.S.C.
BJ2407	<i>MAT<math>\alpha</math> prb1-1122 prc1-407 pep4-3 leu2 trp1 ura3-52</i> <i>MAT<math>\alpha</math> prb1-1122 prc1-407 pep4-3 leu2 trp1 ura3-52</i>	Y.G.S.C.
BJ3501	<i>MAT<math>\alpha</math> pep4::HIS3 prb1-<math>\Delta</math>1.6R his3-<math>\Delta</math>200 ura3-52 GAL can1</i>	Y.G.S.C.
BJ3505	<i>MAT<math>\alpha</math> pep4::HIS3 prb1- 1.6R HIS3 lys2-208 trp1-<math>\Delta</math>101 ura3-52 gal2 can1</i>	Y.G.S.C.
YPH499	<i>MAT<math>\alpha</math> ura3-52 lys2-801 ade2-101 trp1-<math>\Delta</math>63 his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1</i>	Y.G.S.C.
STY99	<i>MAT<math>\alpha</math> ura3-52 lys2-801 ade2-101 trp1-<math>\Delta</math>63 his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1 prb1::TRP1</i>	This study
YW12	<i>MAT<math>\alpha</math> prc::LEU2 leu2-3.112 ura3-52</i>	Yoh Wada

Y.G.S.C.: Yeast Genetic Stock Center. Strain YW12 was kindly provided by Yoh Wada (University of Tokyo).

### Release of Radioactivity from Cells Labeled with [<sup>14</sup>C]Leucine

Cells of strains YPH499 and STY99 were cultured in SD medium with suitable supplements in the presence of 250 MBq/ml of [<sup>14</sup>C]leucine for 20 h to the end of logarithmic phase. They were then collected by centrifugation, washed twice with S(-N) medium, suspended in S(-N) medium at  $2 \times 10^7$  cells/ml, and distributed in two tubes. One tube was supplemented with 1 mM PMSF and the other with 1% ethanol as a control. Starvation culture was carried out in a 30°C incubator with shaking. At 1-h intervals aliquots (150  $\mu$ l) were withdrawn, diluted with 1 ml of the same starvation medium, and promptly filtered on a membrane filter (Millex-GV; Millipore Continental Water Systems, Bedford, MA). The radioactivities in the filtrates were determined with Sintisol 500 (Dojin, Japan) in an Aloka LSC-3000 liquid scintillation counter.

### Reagents

Cycloheximide, 4',6-diamidino-2-phenylindole (DAPI), *N*-ethylmaleimide, PMSF, and quinacrine were purchased from Sigma Chemical Co. (St. Louis, MO). Adenine sulfate, chloramphenicol, leucine, tryptophan, and uracil were from Wako. Zymolyase 20T was from Seikagaku Kogyo (Tokyo, Japan). Ficoll 400 was from Pharmacia Fine Chemicals (Piscataway, NJ). Leupeptin was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Aniline blue was from Chroma (Köngen, Germany). Bafilomycin A<sub>1</sub> was a generous gift from M. Yoshida (Tokyo Institute of Technology, Tokyo, Japan). L-[<sup>14</sup>C]leucine (9287 MBq/mmol) was purchased from ICN Radiochemicals (Irvine, CA). All other reagents used were of analytical grade.

### Results

#### Appearance of Spherical Bodies in Vacuoles in Nitrogen-deficient Conditions

The rationale of this work was that if mutant cells deficient in vacuolar proteinases show an autophagic response to nutrient starvation, cellular components sequestered in the vacuoles should not be degraded, and should accumulate in the vacuoles. To test this possibility, we examined the morphology of vacuoles using the various strains deficient in multiple vacuolar proteases listed in Table I. In nutrient medium YEPD, these mutant cells grew well, and their vacuoles appeared quite similar to those in wild-type cells (Fig. 1, *a* and *b*). These vacuoles were transparent and contained few, if any, detectable particles. When these cells were transferred from YEPD to a nitrogen-deficient medium, SD(-N), they stopped growing. After incubation for 1 h in SD(-N) medium, several spherical bodies that showed ceaseless Brownian movement were seen in the vacuoles of almost all

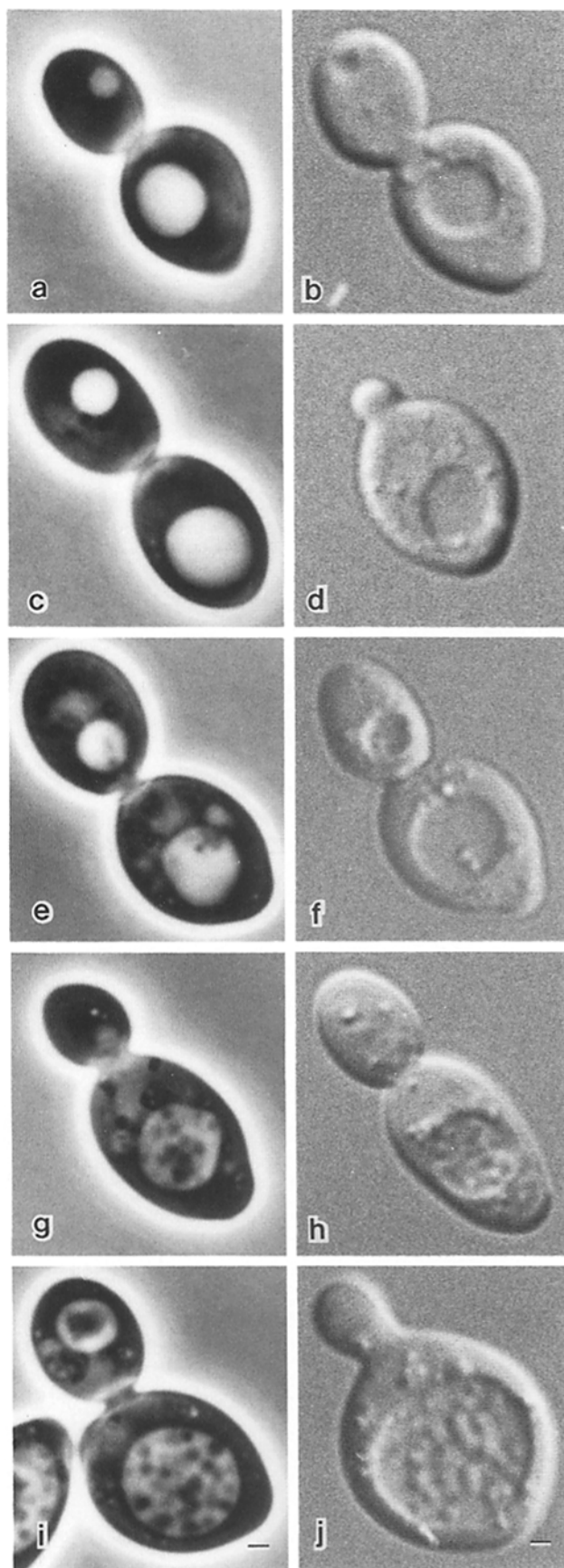
cells (Fig. 1, *e* and *f*). These bodies gradually increased in number (Fig. 1, *g* and *h*) and in 3 h they filled the vacuoles almost completely (Fig. 1, *i* and *j*). At this stage, they were so tightly packed in the vacuoles that their movement was restricted. As the bodies accumulated, the total volume of the vacuolar compartment increased significantly (see Fig. 1). On further incubation, the vacuoles became aberrant shape, and were hardly detectable by light microscopy.

Diploid multiple vacuolar protease-deficient strains do not sporulate in a sporulation medium such as 2% potassium acetate. These cells accumulated the bodies when they were transferred to 2% potassium acetate. However, the accumulation of the bodies is not strictly coupled with sporulation for the following reasons: (*a*) Haploid cells with defects in vacuolar proteases (BJ1991, BJ2168, BJ3501, and BJ3505) also accumulated the bodies in their vacuoles when they were transferred from YEPD to 2% potassium acetate or SD(-N) medium; (*b*) glucose did not interfere with the accumulation of the autophagic bodies in the vacuoles; and (*c*) the  $\rho^o$  strain of BJ926 also actively accumulated the bodies in their vacuoles.

#### Conditions for Accumulation of Spherical Bodies in Vacuoles

Change from YEPD medium to a synthetic medium itself did not induce the accumulation of the bodies if auxotrophic requirements were met (Table II). When BJ2407 cells were transferred to a synthetic medium without amino acids, SD(-A), they also accumulated the bodies, though at a slower rate. Therefore, we examined the effects of depletions of single components. Deficiency of either leucine or tryptophan resulted in the appearance of the bodies in the vacuoles, but the amount of these bodies in the vacuoles differed depending on which amino acid was omitted (Table II). Under these amino acid starvation conditions, the vacuoles never became filled with the bodies, even during prolonged incubation. In contrast, starvation of auxotrophic uracil did not cause any morphological change in the vacuoles, although it stopped cell growth (Table II).

When cells of multiple vacuolar protease-deficient strains were transferred from YEPD medium to SG medium, they stopped growing and accumulated bodies similar to those in a nitrogen-deficient medium. As they cannot use glycerol as a carbon source (unpublished results), they are exposed



**Figure 1.** Morphological changes of vacuoles of multiple vacuolar protease-deficient mutants during nitrogen starvation. BJ2407 cells at different stages during incubation in SD(-N) medium for 0 min

**Table II.** Accumulation of Autophagic Bodies in the Vacuoles of BJ2407 in Different Nutrient-deficient Media

Medium	Deficiency	Accumulation
SD+L+W+Ura	none	-
SD(-N)	nitrogen source	+++
SD(-A)	amino acids and uracil	++
SD(-A)+W+Ura	leucine	++
SD(-A)+L+Ura	tryptophan	+
SD+L+W	uracil	-

L, leucine; Ura, uracil; W, tryptophan. Plus symbols indicate degrees of accumulation of autophagic bodies in the vacuoles and a minus symbol indicates no accumulation.

to carbon starvation in this medium. Similar results were obtained in medium without glycerol. Transfer to a sulfate-deficient medium also induced similar structures in the vacuoles (data not shown). These results indicate that accumulation of the bodies in the vacuoles is a kind of general response of the cells to adverse conditions for growth. It remains to be determined whether there is a common signal to various starvation conditions that induces accumulation of these bodies in the vacuoles.

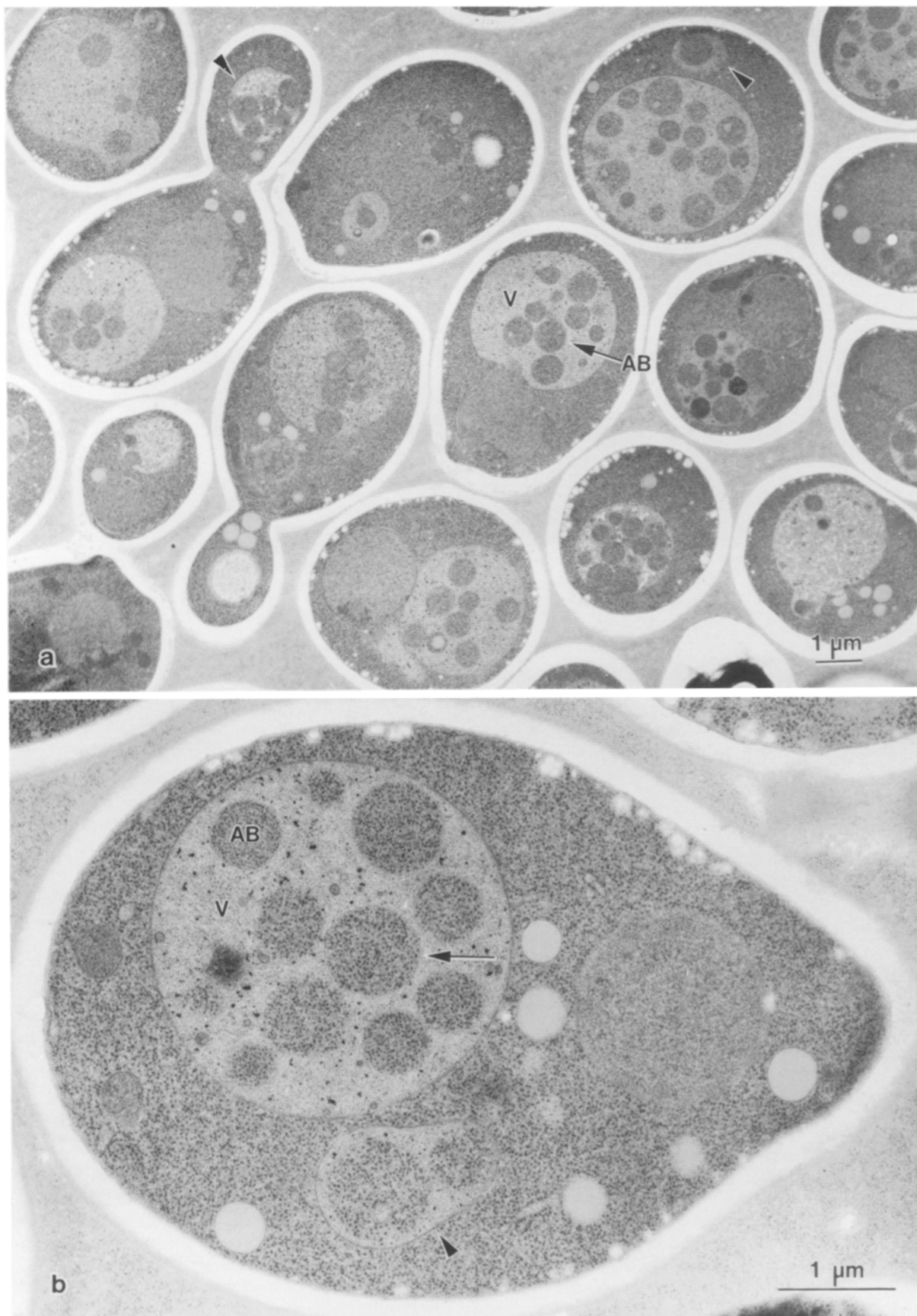
### Electron Microscopic Observation of Nitrogen-starved Cells

To characterize the bodies that accumulated in the vacuoles under nutrient-deficient conditions, we used freeze-substitution fixation methods to prepare cells for electron microscopic examination. Typical images of the starved cells in SD(-N) medium are shown in Fig. 2. In this medium, cell cycle progression soon stopped, and after 2 h almost all the cells had accumulated many spherical bodies, regardless of their stage in the cell cycle. Even small vacuoles in bud and mother portions of dividing cells contained the spherical bodies (Fig. 2, arrowheads). The bodies in the vacuoles were surrounded by a unit membrane which appeared thinner than any other intracellular membrane (Fig. 2 b, arrow). The contents of the bodies were morphologically indistinguishable from the cytosol and included cytoplasmic ribosomes. This indicates that yeast cells have the ability to sequester their own cytosolic components in the vacuoles. Thus we concluded that these spherical bodies in the vacuoles were formed as a result of autophagy, and we named them "autophagic bodies."

### Accumulation of Autophagic Bodies in Carbon-deficient Cells

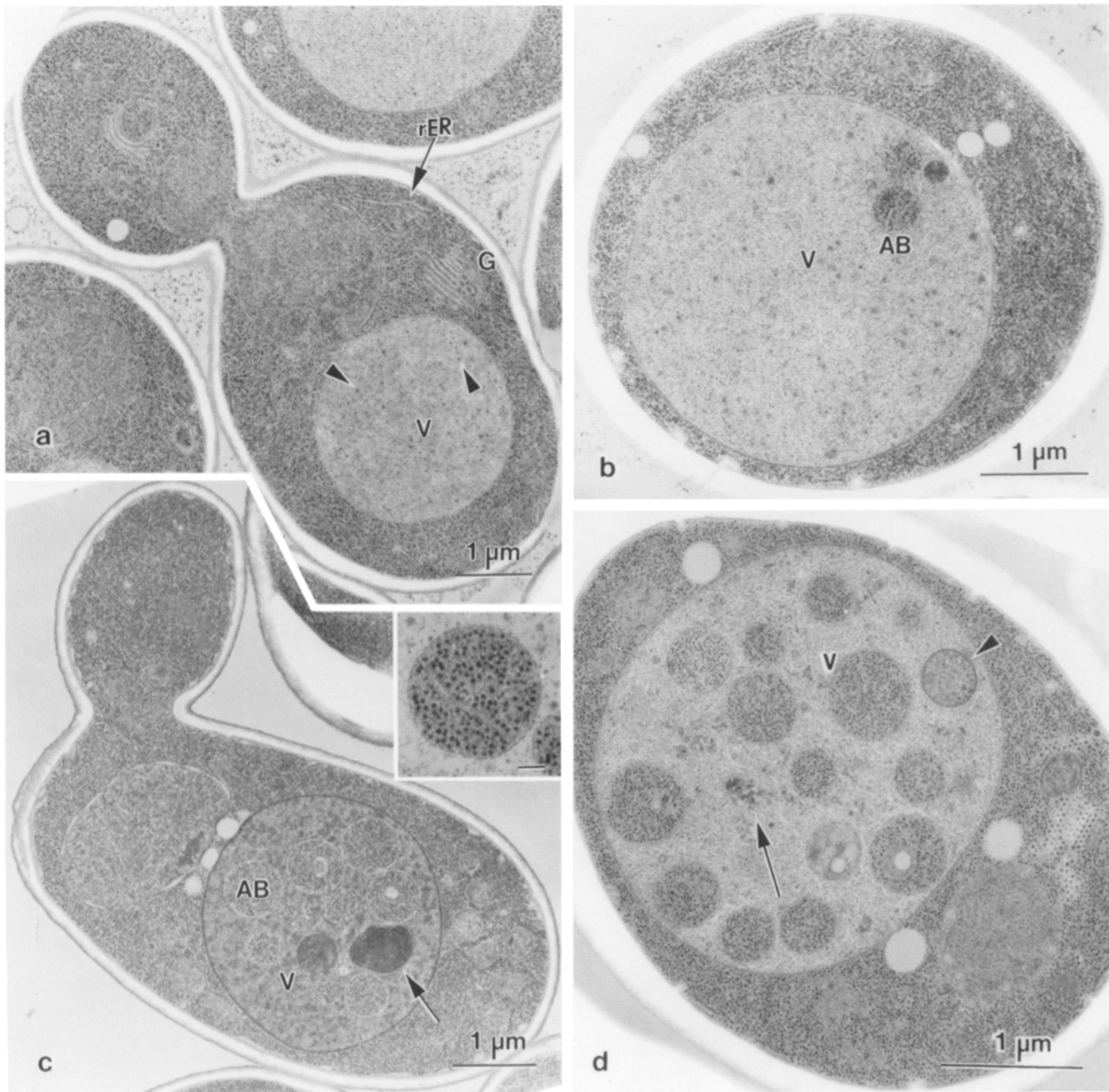
Cellular organelles in the vacuoles of protease-deficient cells grown in YEPD appeared quite normal (Fig. 3 a). Some thin sections showed a well-organized, typical Golgi body with six to eight cisternae. In YEPD, the vacuoles of these cells at all stages of growth contained no detectable structures except small particles (Fig. 3 a, arrowheads). But in SG medium, autophagic bodies of about 400-nm diam appeared within 30 min (Fig. 3 b). After 3-h incubation, the vacu-

(a and b), 15 min (c and d), 1 h (e and f), 2 h (g and h), and 3 h (i and j). Phase contrast (a, c, e, g, and i) and Nomarsky (b, d, f, h, and j) images. The bar represents 1  $\mu$ m. Similar results were obtained with all mutants used.



**Figure 2.** Morphology of vacuoles in multiple protease-deficient cells under nitrogen-starvation. BJ3505 cells incubated for 2 h in SD(-N) medium. (a) At low magnification. (b) A typical cell at high magnification. AB, autophagic body; V, vacuole. Essentially similar images were obtained with all mutants used.





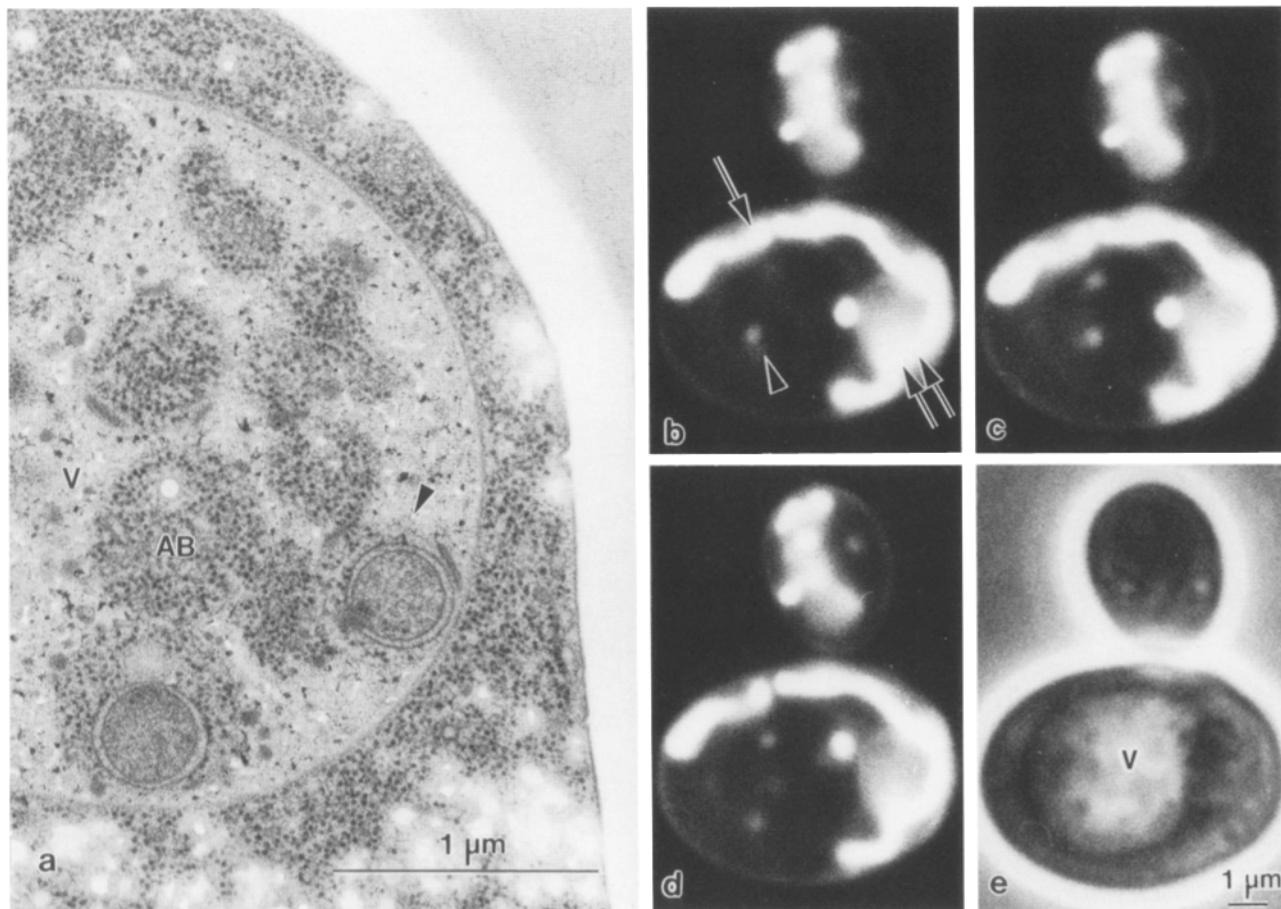
**Figure 3.** Morphological changes of vacuoles in carbon-deficient conditions. BJ926 cells were incubated in SG medium for 0 min (*a*), 30 min (*b*), 3 h (*c*), and 8 h (*d*). The inset in *c* shows an autophagic body containing rough endoplasmic reticulum at higher magnification. The bar represents 100 nm. AB, autophagic body; G, Golgi body; RER, rough endoplasmic reticulum; V, vacuole.

oles were almost filled with autophagic bodies of 400–900-nm diam, which showed no apparent morphological differences from those induced under nitrogen-deficient conditions. After incubation for up to 8 h, most autophagic bodies were still intact, but a few were broken, and their membrane fragments (Fig. 3 *d*, arrow) and ribosomes appeared in the vacuolar sap. The accumulation of autophagic bodies in SG medium was slightly slower than that in SD(-N) medium. Furthermore, under these carbon-deficient conditions, the vacuoles never became packed with the autophagic bodies, which thus continued to show Brownian movement.

### **Contents of Autophagic Bodies in Vacuoles**

Most autophagic bodies contained ribosomes of the same density as cytoplasmic ribosomes, but some contained ribosomes of higher density, suggesting that they had been condensed (Fig. 3 *c*, arrow). At present it is not known when and where this condensation occurs.

Occasionally autophagic bodies enclosed RER (Fig. 3, *b* and *c*, inset), mitochondria (Fig. 3 *d*, arrowhead), and other intracellular structures such as lipid granules, glycogen granules, and membrane vesicles. In some cases they con-



**Figure 4.** Incorporation of mitochondria into vacuoles. (a) Electron micrograph of a BJ926 cell incubated for 2 h in SD(-N) medium. (b-d) Fluorescence micrographs of BJ926 cells incubated for 5 h in SD(-N) medium. Cells were stained with DAPI. The three photographs were taken at the same focal plane. The position of the nuclear DNA is indicated by double arrows. (e) Phase contrast image of the same cell. V, vacuole.

tained a mitochondrion only (Fig. 3 d, arrowhead), but in other cases they contained cytosol as well (Fig. 4 a, arrowhead). Incorporation of mitochondria into the vacuoles was also demonstrated by vital staining with DAPI. In normally growing cells, stained mitochondrial DNA usually appeared as strings of beads (Fig. 4 b, arrow) and dots (Fig. 4 b, arrowhead). When these cells were incubated in SD(-N) medium, their mitochondrial DNA appeared as a coalesced form with several dots. During starvation, dots of mitochondrial DNA appeared in the vacuoles (Fig. 4, b, c, and d). These dots of mitochondrial nucleoid incorporated into vacuoles were easily distinguishable as they showed active Brownian movement. After incubation for several hours, most cells had four to six dots of mitochondrial nucleoid in their vacuoles, and some had up to 10 dots. These results indicate that a significant portion of the mitochondria was sequestered in vacuoles under nitrogen-starvation conditions to be degraded.

During extensive electron microscopic observations we obtained no indication that membranous organelles were preferentially sequestered in the vacuoles. Thus the material sequestered in the vacuoles for degradation under adverse conditions seemed to be mainly cytosol itself, including dense ribosomes and many housekeeping enzymes.

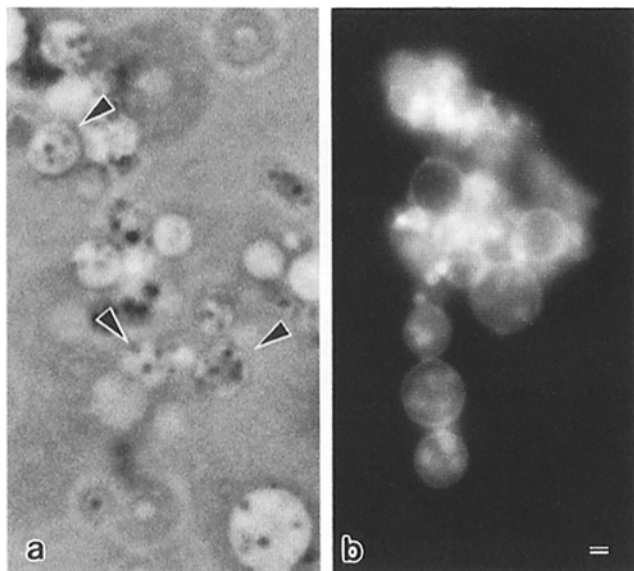
### Mutation Involved in Accumulation of Autophagic Bodies in Vacuoles

Similar results to the above were obtained with various multiple proteinase-deficient strains. To determine the defective vacuolar enzyme(s) responsible for the accumulation of the autophagic bodies in the vacuoles, we crossed a multiple vacuolar protease-deficient mutant (BJ3505) with the wild type cells and constructed mutants lacking a single vacuolar

**Table III.** Defect in Vacuolar Protease Responsible for Accumulation of Autophagic Bodies in the Vacuoles

Strains	Genotype	Accumulation
KTY1-3A	<i>pep4 PRB1 PRC1</i>	yes
KTY1-3B	<i>pep4 prb1 PRC1</i>	yes
KTY1-3C	<i>PEP4 prb1 PRC1</i>	yes
KTY1-3D	<i>PEP4 PRB1 PRC1</i>	no
YW-12	<i>PEP4 PRB1 prc1</i>	no

KTY1-3A to 3D are originated from a tetrad of the diploid obtained by crossing BJ3505 with X2180-1B. All cells were grown in YEPD and transferred to SD(-N) or SG. Their accumulation of autophagic bodies was observed after 4 h.



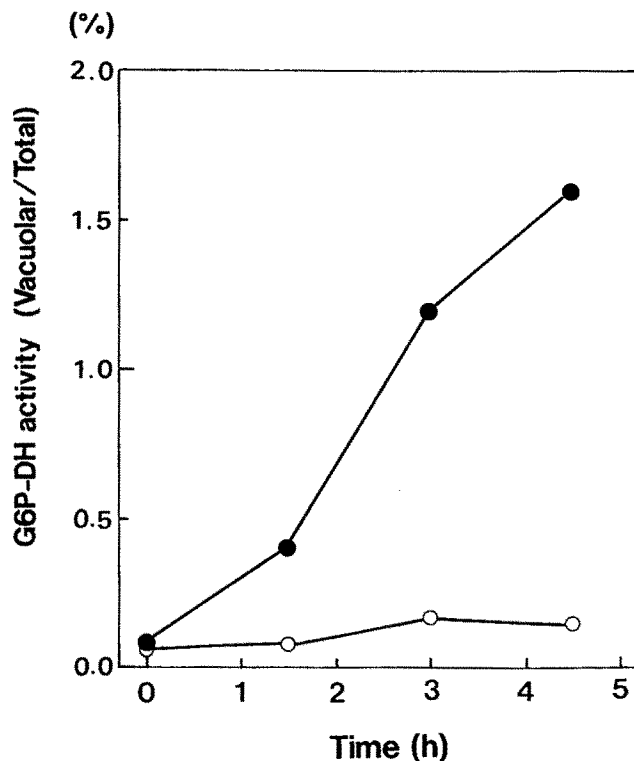
**Figure 5.** Isolated vacuoles containing autophagic bodies. Vacuoles were isolated from BJ3505 cells incubated in SG for 4 h. (a) Phase contrast image, (b) fluorescence image of the vacuoles stained with quinacrine. Similar preparations were obtained with STY499 and other multiple protease-deficient cells. Bar, 1  $\mu$ m.

protease. Examinations of 10 tetrads showed that cells lacking either proteinase A (PrA) or proteinase B (PrB) accumulated the autophagic bodies. Typical results of tetrad analysis are shown in Table III. KTY1-3A and 3C, which are disruptants of the *PEP4* (*PRA1*) and *PRB1* gene, respectively, both accumulated autophagic bodies in the vacuoles, but YW12, with a disrupted *PRC1* accumulated scarcely any autophagic bodies. These results indicate that CPY itself is not involved in this phenomenon. A mutation of *PEP4*, which encodes PrA, causes pleiotropic defects in other vacuolar enzymes (Jones, 1984), because PrA is required to process inactive precursors of various vacuolar enzymes including PrB. Therefore, the accumulation of autophagic bodies in the vacuoles is probably the consequence of the absence of PrB activity.

For confirmation of this conclusion, we disrupted the *PRB1* gene of YPH499 by the standard method and constructed the STY99 strain. YPH499 and its *PRB1* disruptant (STY99) were incubated for 3 h in various starvation conditions. STY99 cells accumulated the autophagic bodies in exactly the same manner as did multiple protease-deficient cells, but no accumulation was observed in the vacuoles of the YPH499 cells (data not shown). Thus we conclude that lack of the *PRB1* product, PrB, is sufficient for accumulation of autophagic bodies in the vacuoles. In further biochemical analyses, we mainly used STY99 cells.

#### Biochemical Analysis of Autophagic Bodies

To characterize the autophagic bodies further, we isolated vacuoles from starved cells. Under nitrogen-deficient conditions, the vacuoles lost their spherical contour and were difficult to isolate. But under carbon-starved conditions, the vacuoles remained round and could be isolated in good yield by a modification of the method of Ohsumi and Anraku (1981). These isolated vacuoles contained intact autophagic bodies, as shown in Fig. 5 (arrowheads). Quinacrine-stained

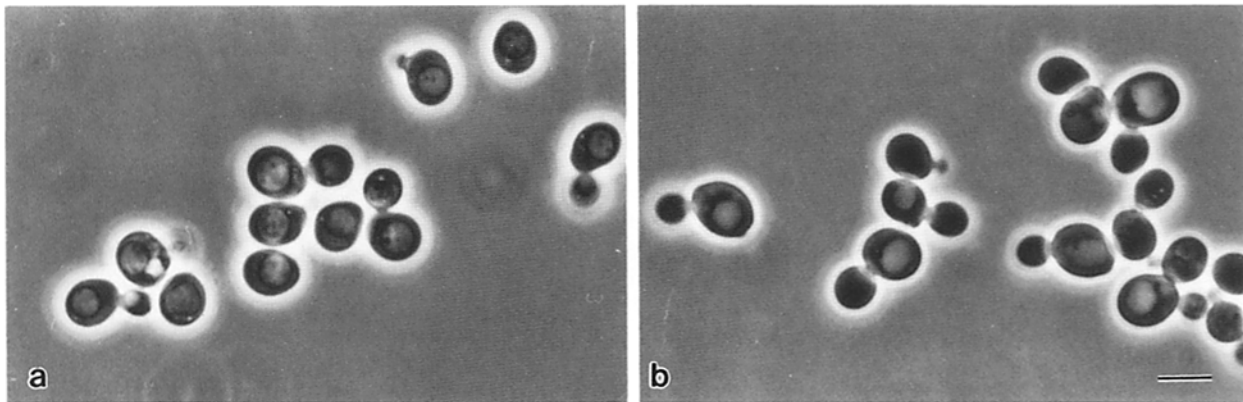


**Figure 6.** Appearance of activity of the cytosolic enzyme G6PDH in isolated vacuoles. The latent G6PDH activity in the vacuoles is presented as the total activity in a spheroplast lysate. (—●—) The latent activity in the vacuoles prepared from STY99 (*prb1::TRP1*). (—○—) The latent activity in the vacuoles from YPH499 (*PRB1*).

vacuolar and nuclear membranes and autophagic bodies in cells incubated in SG medium. Isolated vacuolar membranes and autophagic bodies were also stained with quinacrine. Using fluorescence microscopy, we found that isolated vacuoles contained 5–20 autophagic bodies. As the method used for vacuole isolation depended on flotation, we may have collected vacuoles containing fewer autophagic bodies preferentially.

The isolated whole vacuoles from starved cells showed only slight activity of the marker enzyme of the cytosol, G6PDH. However, appreciable G6PDH activity was found in the preparation after sonication or freezing thawing. This latent activity of G6PDH increased during starvation as shown in Fig. 6 (closed circles). Assuming that the volume of a single autophagic body (500 nm) is roughly 0.1% of that of the cytosol, the activity of G6PDH corresponds well with the total volume of autophagic bodies incorporated into isolated vacuoles. On the contrary, vacuoles from wild type cells did not accumulate the enzyme activity during starvation (Fig. 6, open circles), suggesting that latent G6PDH is located in the autophagic bodies. Further, the time course of accumulation of material with absorption at 260 nm, most of which was due to cytoplasmic ribosomes, was similar (data not shown). These data are consistent with those obtained by electron microscopic analysis of accumulation of cytoplasmic ribosomes in the autophagic bodies. Preliminary experiments showed that the specific activity of G6PDH in the vacuoles of the starved cells was similar to that in the cytosol, suggesting that at least one cytosolic enzyme was sequestered in vacuoles in an intact form.





**Figure 7.** Accumulation of autophagic bodies in wild-type cells in the presence of PMSF. (a) YPH499 cells grown in YEPD were transferred to SG medium containing 1 mM PMSF for 4 h. (b) Then the cells were collected and resuspended in fresh YEPD medium for 4 h. Bar, 5  $\mu\text{m}$ .

### **Induction of Autophagic Bodies in Vacuoles of Wild-Type Cells**

PrB is a serine proteinase and several specific inhibitors of this enzyme have been reported. The oligopeptide leupeptin, which is an inhibitor of serine proteinases had no effect on the proteinases in intact wild-type cells. Moreover, a much higher concentration of leupeptin applied to the cells to introduce it into vacuoles by endocytosis, as shown by Riezman (1985), also did not cause accumulation of autophagic bodies.

When wild-type cells grown in YEPD were suspended in SD(-N) medium containing 1 mM PMSF, however, autophagic bodies appeared in their vacuoles in exactly the same manner as in *prb1* or multiple protease-deficient mutants (Fig. 7 a). They filled the vacuoles within 4 h and remained in the vacuoles stably. On electron microscopic examination, these autophagic bodies induced by PMSF in wild-type cells were indistinguishable in morphology from those accumulated in protease-deficient cells. Accumulation of these autophagic bodies could be induced in wild-type cells simply by adding PMSF to the medium.

If the autophagic bodies are an intermediate in the normal autophagic process, they should be degraded rapidly in *PRB1* cells. To confirm this, we examined the effect of restoration of PrB activity on accumulated autophagic bodies in the vacuoles. The effect of PMSF is known to be irreversible, so we transferred cells that had accumulated autophagic bodies in the presence of PMSF to fresh YEPD medium without PMSF. In these cells, autophagic bodies disappeared completely within 3 to 4 h (Fig. 7 b). During this time, the cells divided twice at most, so the rapid disappearance of autophagic bodies could not be explained simply by dilution. This finding supports the idea that the autophagic bodies are degraded in the vacuoles and that they are a normal intermediate form of cytosol sequestered in vacuoles to be degraded.

### **Estimation of Protein Degradation during Starvation**

Protein degradation in vivo was measured as release of radioactivity from cells labeled with [ $^{14}\text{C}$ ]leucine into the medium. When uniformly labeled wild-type cells (YPH499) were incubated in SG(-N) or SG medium, radioactivity was gradually released into the medium (Fig. 8 a). The release

from the *prb1* cells (STY99) was significantly less. Furthermore, release of radioactivity from YPH499 was sensitive to PMSF, while that from STY99 was not, and the release from YPH499 in the presence of PMSF coincided with that from STY99 (Fig. 8, a and b). These results support the following conclusions: (a) Carbon-starvation induces protein degradation, and liberation of part of the amino acids (or their derivatives) from the cells; (b) this protein degradation depends entirely on PrB activity; and (c) the main effect of PMSF on wild-type cells is inhibition of PrB activity in the vacuoles. Release of radioactivity from the cells was less in SD(-N) medium than in SG medium, possibly because of retention of amino acids within the cells in the presence of an energy source. These results indicate that the main pathway of protein degradation in yeast under adverse conditions is PrB-dependent, suggesting that autophagy in vacuoles is of primary importance in protein turnover under these conditions.

### **Effects of Various Reagents on Accumulation of Autophagic Bodies in Vacuoles**

The effects of various reagents on the accumulation of autophagic bodies under nutrient-deficient conditions were tested. De novo synthesis of proteins is a prerequisite for accumulation of the autophagic bodies, because their accumulation was completely inhibited by addition of 25  $\mu\text{g}/\text{ml}$  of cycloheximide at the beginning of starvation. In contrast, 50  $\mu\text{g}/\text{ml}$  of chloramphenicol did not affect the accumulation of autophagic bodies in the vacuoles of multiple vacuolar protease-deficient cells (Table IV).

*N*-Ethylmaleimide (NEM) is reported to inhibit transport of protein between the Golgi stacks and also various membrane fusion events (Malhotra et al., 1988; Wilson et al., 1989). Addition of 0.1 mM NEM to starvation medium completely abolished the accumulation of autophagic bodies in the vacuoles (Table IV), but did not affect cell viability. Thus, there must be one or more NEM-sensitive steps in the process of autophagy in yeast cells.

The cytosolic  $\text{Ca}^{2+}$  concentration of the cells was raised by treatment with the calcium ionophore A23187. Addition of A23187 had no effect on the accumulation of autophagic bodies in the vacuoles (Table IV).

When BJ926, BJ2407, BJ3505, or STY99 cells were incubated in SD(-N) medium containing quinacrine, this dye

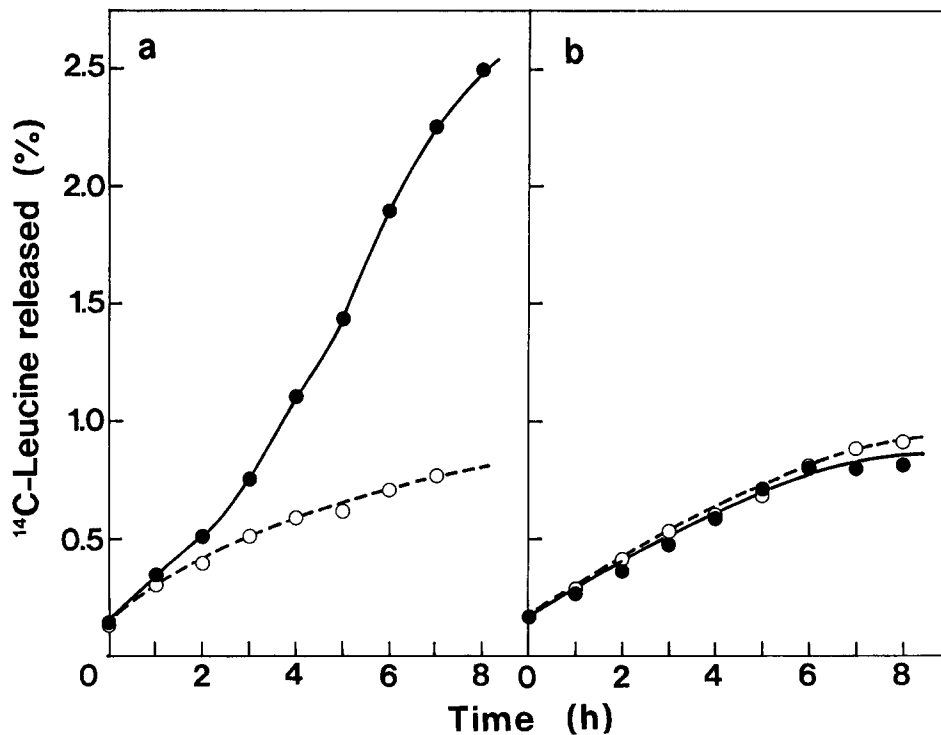


Figure 8. PrB-dependent release of radioactivity from cells labeled with [ $^{14}\text{C}$ ]leucine. Release of radioactivity from YPH499 (a) or STY99 (b) into the medium was measured as described in Materials and Methods. With 1 mM PMSF (---○---) with 1% ethanol (—●—).

was concentrated in the vacuoles, indicating the maintenance of a pH gradient across the vacuolar membrane during nitrogen starvation. Dissipation of the pH gradient across the vacuolar membrane has been reported to cause missorting of vacuolar enzymes (Rothman et al., 1989). Bafilomycin  $A_1$  is a potent inhibitor of vacuolar type  $\text{H}^+$  ATPases in fungi, animal cells, and plant cells (Bowman et al., 1988), and in the presence of  $10 \mu\text{M}$  bafilomycin  $A_1$ , staining of the yeast vacuoles with quinacrine was completely abolished (data not shown). However, the accumulation of autophagic bodies proceeded normally in the presence of  $10 \mu\text{M}$  bafilomycin  $A_1$  (Table IV). Thus acidification of the vacuoles is not necessary for the accumulation of autophagic bodies. This conclusion was confirmed using 200 mM ammonium acetate (Table IV).

## Discussion

In this paper, we demonstrated that yeast cells lacking vacuolar proteases show extensive accumulation of autophagic bodies under various adverse environmental conditions. Experiments on the effects of gene disruption and inhibitors

showed that a defect in PrB is responsible for this event. Moreover using PMSF, we demonstrated the accumulation of autophagic bodies in wild-type cells and found that these autophagic bodies disappeared rapidly from the vacuoles on restoration of PrB activity. Furthermore, when wild-type cells were transferred from YEPD to nutrient-deficient medium, a few autophagic bodies were observed transiently in 1–2 h, but further accumulation did not occur. These facts suggest that the autophagic bodies are degraded rapidly in wild-type cells.

To characterize these autophagic bodies in the vacuoles, we used rapid freezing and freeze-substitution fixation methods for their examination. Vacuolar sap is like an aqueous salt solution, so it was very difficult to achieve good preservation of inclusions floating in it. But satisfactory preparations were obtained by cryo-fixation and freeze-substitution fixation. These techniques also resulted in greatly improved preservation of endomembrane cisternae and cytoplasmic ribosomes, which provided the most reliable marker of the cytosol. The mechanism of formation of autophagic bodies was not addressed in this work, but preliminary studies suggested that autophagosomes may be formed in the cytoplasm and subsequently fuse with the vacuolar membrane (Baba, M., et al., manuscript in preparation).

One problem about autophagy is whether materials are sequestered in lysosomes or vacuoles selectively or nonselectively. In yeast, the contents of the autophagic bodies are indistinguishable from the cytosol itself, at least by EM. Some autophagic bodies were found to contain RER, small vesicles, mitochondria, and so on. However, these membranous organelles did not seem to be accumulated in the autophagic bodies selectively, as reported in some cases of autophagy in rat liver (Seglen, 1987), although quantitative analysis is required to confirm this.

Yeast cells grown in a nutrient medium such as YEPD may

Table IV. Effects of Various Reagents on the Accumulation of Autophagic Bodies in the Vacuoles

Reagent	Accumulation
50 $\mu\text{g}/\text{ml}$ Chloramphenicol	yes
25 $\mu\text{g}/\text{ml}$ Cycloheximide	no
200 mM $\text{CH}_3\text{COONH}_4$	yes
0.1 mM NEM	no
10 $\mu\text{M}$ A23187	yes
10 $\mu\text{M}$ Bafilomycin $A_1$	yes

Cells of BJ926, BJ2407, and BJ3505 were tested.

be adapted to rapid growth, and may obtain nutrients from the medium. In these conditions, the cytosol of the cells contains numerous ribosomes, and high levels of housekeeping enzymes such as those for energy supply (fermentation) and protein synthesis. When these cells are shifted to a medium that does not support growth, they may degrade ribosomes and housekeeping enzymes to adapt to the adverse environment. The number of ribosomes depends on the growth medium, and decreases significantly (>40%) in the early stage of sporulation (Hopper et al., 1974). The sporulation process is triggered by nitrogen starvation, so this cell differentiation must depend entirely on the degradation of preexistent proteins. The present results strongly suggest that protein degradation under various starvation conditions is mediated by autophagy and that a defect in the normal process of degradation of cellular components in the vacuoles may be the reason for the sporulation-negative phenotype of vacuolar proteinase mutants. Yeast cells are known to enter the G<sub>1</sub> phase of the cell cycle when they are exposed to nitrogen and sulfate deficient conditions. However, strains lacking the multiple vacuolar proteases could not progress through the cell cycle under these conditions. Thus the proteolysis in the vacuoles must be the main route to supply the materials necessary for process through the cell cycle under adverse circumstances.

Autophagy, which may involve many steps, must be strictly controlled. The most promising approach to elucidate the molecular mechanism of the whole process of autophagy might be its genetic dissection into elementary steps. The experimental procedure to induce autophagy reported in this paper is simple: autophagy can be induced synchronously in almost all cells by placing them in nutrient-deficient media. The accumulation of autophagic bodies at the phagolysosomal stage of vacuoles can be detected by light microscopy. This means that the occurrence of autophagy can be judged by the appearance of autophagic bodies in the vacuoles. Thus mutants with defects in the autophagic process can easily be identified and isolated. Vacuoles in yeast are much more homogeneous than lysosomes in animal cells and are easily isolated. In addition to genetic studies, biochemical and molecular biological approaches may be helpful in understanding the whole process of autophagy at the molecular level.

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