Ultrastructural and Biochemical Characterization of Autophagy in Higher Plant Cells Subjected to Carbon Deprivation: Control by the Supply of Mitochondria with Respiratory Substrates

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Abstract. Autophagy triggered by carbohydrate starvation was characterized at both biochemical and structural levels, with the aim to identify reliable and easily detectable marker(s) and to investigate the factors controlling this process. Incubation of suspension cells in sucrose-free culture medium triggered a marked degradation of the membrane polar lipids, including phospholipids and galactolipids. In contrast, the total amounts of sterols, which are mainly associated with plasmalemma and tonoplast membranes, remained constant. In particular, phosphatidylcholine decreased, whereas phosphodiesters including glycerylphosphorylcholine transiently increased, and phosphorylcholine (P-Cho) steadily accumulated. P-Cho exhibits a remarkable metabolic inertness and therefore can be used as a reliable biochemical marker reflecting the extent of plant cell autophagy. Indeed, whenever P-Cho accumulated, a massive regression of cytoplasm was noticed using EM. Double membrane-bounded vacuoles were formed in the peripheral cytoplasm during sucrose starvation and were eventually expelled into the central vacuole, which increased in volume and squeezed the thin layer of cytoplasm spared by autophagy.

The biochemical marker P-Cho was used to investigate the factors controlling autophagy. P-Cho did not accumulate when sucrose was replaced by glycerol or by pyruvate as carbon sources. Both compounds entered the cells and sustained normal rates of respiration. No recycling back to the hexose phosphates was observed, and cells were rapidly depleted in sugars and hexose phosphates, without any sign of autophagy. On the contrary, when pyruvate (or glycerol) was removed from the culture medium, P-Cho accumulated without a lag phase, in correlation with the formation of autophagic vacuoles. These results strongly suggest that the supply of mitochondria with respiratory substrates, and not the decrease of sucrose and hexose phosphates, controls the induction of autophagy in plant cells starved in carbohydrates.

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Classical autophagy is a general mechanism whereby eucaryotic cells degrade parts of their own cytoplasm, including organelles (except the nucleus). This process has been well documented in the animal kingdom (for reviews see Marzella and Glaumann, 1987; Seglen et al., 1989) and has been implicated, for instance, in the nonselective bulk degradation of proteins triggered by nutrient deprivation. Most of the work on autophagy in animal cells is related to proteolysis in liver, using organs or isolated cells perfused with controlled medium. Structural and biochemical studies have indicated that portions of cytoplasm are first sequestered by a double membrane-bound vacuole (autophagosome), originating from ribosome-free regions of the RER (Dunn, 1990a). This nascent autophagic vacuole is loaded with various acid hydrolases by fusion with preexisting lysosomes or Golgi apparatus–derived vesicles to form a single membrane–bound degradative vacuole or autolysosome (Dunn, 1990b), the loss of the inner membrane being presumably due to hydrolysis or fusion to the outer membrane. Evidence suggests that autophagy in animal cells is regulated by physiological effectors such as amino acids or hormones (for review see Grinde, 1985). Recently, autophagy has also been characterized in *Saccharomyces cerevisiae* transferred to a synthetic medium devoid of nutrients (Takeshige et al., 1992; Baba et al., 1994). Autophagy-defective mutants have been isolated (Tsukada and Ohsumi, 1993), and their characterization will probably allow a further understanding of the autophagic process and its regulation in yeasts.

Autophagy has been documented in higher plant cells by a number of morphological, cytochemical, and cell fractionation studies (for reviews see Matile, 1975, 1987; Marty et al., 1980; Herman, 1994). Portions of cytoplasm are sequestered and further degraded by lytic vacuolar enzymes. Two types of autophagic processes have been de-
phosphorylethanolamine. The formation of the vacuole in differentiating vegetative cells (Marty, 1973, 1978) resembles the autophagy described. The formation of the vacuole in differentiating cells from cotyledons of germinating seedlings (Van der Wilden et al., 1980; Herman et al., 1981; Melroy and Herman, 1991). On the basis of morphological criteria (showing alteration of the sequestered material), in situ localization of hydrolase activities, and enzymatic assays of isolated vacuolar fractions, it is assessed that digestion of the sequestered material proceeds in the preexisting vacuole. However, there are few reports on the biochemical characterization of autophagic processes. Germinating mung bean cotyledons apparently degrade membranes by autophagy with a net loss of phospholipids (Van der Wilden et al., 1980). Similarly, proteins from the vacuole membrane are degraded by autophagy during germination (Nishimura and Beevers, 1979; Melroy and Herman, 1991). Interestingly, autophagy in plant, animal, and yeast systems is often associated with nutrient starvation.

In previous publications we have shown that transfer of sycamore cells in sucrose-free medium triggered the following cascade of events (for review see Douce et al., 1991): (a) the consumption of initial carbohydrate reserves and the growth arrest; (b) the marked decline in the uncoupled rate of respiration; (c) the breakdown of proteins and concomitant accumulation of free amino acids; and (d) the decline in many enzymatic activities involved in glycolysis. Carbohydrate starvation is a stress that often occurs in plants. Indeed, constant variations of environmental factors, such as light, water, or temperature, limit the efficiency of photosynthesis and thus reduce the supply of carbohydrates that represent the main respiratory substrates in plant cells (ap Rees, 1990). Using sycamore suspension cells as a model, Dorne et al. (1987) first demonstrated that sugar starvation led to polar lipid hydrolysis. Fatty acids thus released, as well as amino acids, are likely used as oxidizable substrates in place of sugars. Furthermore, the decline of respiration in carbohydrate-starved cells was attributed to a progressive diminution of the number of mitochondria per cell, based on the demonstration of the decline of cardiolipin (diphosphatidylglycerol) and cytochrome aa3 (cytochrome oxidase), two specific mitochondrial markers (Journet et al., 1986). This result suggested that an autophagic process triggered by carbohydrate starvation could be involved in the regression of cytoplasm including organelles like mitochondria (Journet et al., 1986; Douce et al., 1991). In addition, 31P-nuclear magnetic resonance (NMR) studies revealed that carbohydrate deprivation also triggered an accumulation of phosphorylcholine (P-Cho) in the cytoplasmic compartment of starved cells (Roby et al., 1987). It could derive from phosphorylcholine degradation, but it could also originate from further de novo synthesis during the arrest of incorporation into membrane systems of nongrowing starved cells.

However, structural evidence for an autophagy occurring during carbohydrate starvation remains scarce. Likewise, the regulation of the process is unknown. James et al. (1993) suggested that the induction of proteolytic activities involved in the massive breakdown of proteins in glucose-starved maize root tips was controlled by the level of sugars, which rapidly declines during starvation. This idea is supported by the fact that the transcription of many plant genes has been shown to be regulated by sugars through catabolite repression (for review see Sheen, 1994), a mechanism well documented in bacteria (Ullmann, 1985) and yeasts (Gancedo, 1992).

Therefore, a better understanding of autophagy in higher plant cells requires an integrated study in which morphological descriptions are complemented with physiological and biochemical data. The aim of our work was: (a) to better characterize the autophagic processes triggered by carbohydrate starvation, at both biochemical and structural levels, using as a model isolated cells cultivated in controlled conditions; (b) to identify reliable and easily detectable marker(s) of autophagy; and (c) to use these markers for investigating the factors involved in the induction of this process.

In this article, using EM we demonstrate the presence of a very active autophagic activity during sucrose starvation in sycamore cells, leading to a massive degradation of cytoplasm. This process is paralleled with a massive breakdown of membrane lipids except sterols. P-Cho, proposed as a reliable biochemical marker of autophagy and its extent, is used to investigate the control of autophagy by the supply of mitochondria with respiratory substrates.

**Materials and Methods**

**Materials**

Cell suspensions were chosen as a model in which incubation conditions can be easily controlled. Sycamore cells (Acer pseudoplatanus L.) used in the present study were grown at 20°C as a suspension in liquid nutrient media containing sucrose (Bligny and Leguay, 1987). The culture medium was kept at a volume of 0.3 liters and stirred continuously at 60 rpm. Under these conditions, the cell number doubling time was 40-48 h after a lag phase of ~2 d, and the maximum density of sycamore cells was attained after 7-8 d of growth, when the stationary phase is attained. The cell suspensions were maintained in exponential growth by subculturing every 7 d. The fresh weight of cells was measured after staining culture aliquots onto a glass-fiber filter.

Cells harvested from the culture medium were rinsed three times by successive resuspensions in fresh culture medium devoid of sucrose and incubated at zero time into flasks containing sucrose-free culture medium. The fresh weight of cells (per ml of culture) remained constant during the course of carbohydrate starvation (as growth was stopped, see Dorne et al., 1987), so it was used as a reference for quantitative comparisons between control and starved cells.

**Polar Lipid and Sterol Analysis**

Cell suspension aliquots were partially dried by filtration under vacuum and fixed by adding boiling ethanol. Cell lipid extraction and polar lipid determinations were carried out essentially as described by Douce and Jo-

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1. Abbreviations used in this paper: GPC, glycerylphosphorylcholine; GPE, glycerylphosphorylethanolamine; NMR, nuclear magnetic resonance; NTP, nucleosides triphosphate; P-Cho, phosphorylcholine; P-EA, phosphorylethanolamine.
yward (1980a). Fatty acid methyl esters were made by transesterification of polar lipids at 70°C for 1 h in a mixture of methanol/sulfuric acid/benzene (100:0.5:5, vol/vol/vol). Methylesters were extracted with hexane and chromatographed on a Infinito gas chromatograph (GC 1B; Industrie Laboratoire Service, Lyon, France) equipped with a hydrogen flame ionization detector and Infinati integrator (IRC 1B; Industrie Laboratoire Service). Separations were carried out at 175°C using a column packed with 10% diethylene glycol sulfonate on a Varaport 30 chromosorb.

Sterols were extracted in the same way as polar lipids and determined according to Hartmann and Benveniste (1987).

**In Vitro 31P-NMR Measurements**

**Perchloric Extract Preparation.** For perchloric acid extraction, cells (9 g fresh weight) were quickly frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle with 1 ml of 70% (vol/vol) perchloric acid. The frozen powder was then placed at −10°C and thawed. The thick suspension thus obtained was centrifuged at 15,000 g for 10 min to remove particulate matter, and the supernatant was neutralized with 2 M KHCO3 to pH 5. The supernatant was then centrifuged at 10,000 g for 10 min to remove KCIO4; the resulting supernatant was lyophilized and stored in liquid nitrogen. This freeze-dried material was redissolved in 2.5 ml water containing 10% D2O, neutralized to pH 7.5, and buffered with 50 mM Hepes. Divalent cations (particularly Mn2+ and Mg2+) were chelated by the addition of sufficient amounts of 1,2-cyclohexylenedinitrilotetraacetic acid ranging from 30 to 100 µmol depending on the samples.

**NMR Measurements.** Spectra of neutralized perchloric acid extracts were recorded on an NMR spectrometer (AMX 400, wide bore; Bruker Instruments, Inc., Billericia, MA) equipped with a 10-mm multinuclear probe tuned at 162 MHz. The deuterium resonance of D2O was used as a lock signal. Acquisition conditions were: 70° radio frequency pulses (15 µs) at 3.6-s intervals; spectral width 8200 Hz; 1024 scans; Walz-16 1H decoupling sequence (with two levels of decoupling: 1 W during acquisition time, 0.5 W during delay). Free induction decays were induction decays were collected as 8K data points zero filled to 16K and processed with a 0.2-Hz exponential line broadening. 31P-NMR spectra are referenced to methylene diphosphonic acid, pH 8.9, at 16.38 ppm.

**Identifications and Quantifications.** Spectra of standard solutions of known compounds at pH 7.5 were compared with that of a perchloric acid extract of sycamore cells. The definitive assignments were made after running a series of spectra obtained by addition of the authentic compounds to the perchloric acid extracts, according to previous publications (see Roby et al., 1987; Aubert et al., 1994). To accurately determine the total amount of the most abundant soluble organic compounds present in the perchloric extracts, we proceeded as follows: (a) 20-s recycling time used to obtain fully relaxed spectra; and (b) a calibration of the peak intensities by the addition of known amounts of the corresponding authentic compounds. In addition, we took into account the extraction rate of the perchloric extraction. By adding known amounts of authentic compounds to the frozen cells before grinding, we estimated that the overall yield of recovery was 75–80%.

**In Vivo 31P-NMR Measurements**

To get a better signal-to-noise ratio, an experimental arrangement was realized to analyze the maximum cell volume and to optimize the homogeneity of the cell incubation conditions (Roby et al., 1987). Spectra were recorded on a spectrometer (AMX 400, wide bore; Bruker Instruments, Inc.) equipped with a 25-mm probe tuned at 161.93 MHz. Acquisition conditions were: 50° radio frequency pulses (70 µs) at 0.6-s intervals; spectral width 9800 Hz; 6000 scans; Walz-16 1H decoupling sequence (with two levels of decoupling: 2.5 W during acquisition time, 0.5 W during delay). Free induction decays were collected as 4K data points zero filled to 8K and processed with a 0.2-Hz exponential line broadening. 31P-NMR spectra are referenced to methylene diphosphonic acid, pH 8.9, at 16.38 ppm.

**Regression of Membrane Lipids and Evolution of Phosphorous Metabolites in Sycamore Cells during Sucrose Starvation**

In sycamore cell membranes, phosphatidylethanolamine and phosphatidylethanolamine are the major phosphoglycerides, representing up to 80% of the total phospholipids (2.3 and 1.1 µmol per g cell wet weight, respectively) (see Roby et al., 1987). The use of fatty acids deriving from cell membrane polar lipids after a long period of sucrose starvation (see Dorne et al., 1987) should lead, therefore, to the intracellular accumulation of phosphorylated compounds deriving from polar lipid catabolism. Fig. 1 shows representative in vitro 31P NMR spectra (perchloric acid extracts, expanded scale from 0 to 5 ppm) of sycamore cells cultivated with sucrose (bottom spectrum) or starved with carbohydrate for various times (12, 24, 48, 72, and 120). In vivo spectra corresponding to control cells and 48 h–starved cells are shown in Fig. 2. Carbohydrate starvation triggered several noticeable changes.

First, the total amount of glucose-6-P (at 4.56 and 4.52 ppm in extracts), mannose-6-P (at 4.45 and 4.43 ppm in extracts), fructose-6-P (at 3.95 ppm in extracts), and UDP-glucose (at −11.2 and −12.8 ppm in vivo) (Fig. 2) dropped sharply, in agreement with previous observations (Roby et al., 1987). Concomitantly, Fig. 2 shows that cytoplasmic and vacuolar Pi increased considerably, reflecting phosphorymoester hydrolysis (Rébéillé et al., 1985). Fig. 2 also shows that the level of soluble nucleotides triphosphate (NTP) was not significantly modified after 2 d of starvation, indicating that the decline of NTP is not involved in the induction of the autophagic process, as has been suggested for animal cell autophagy (Schellens et al., 1988).

Parenthetically, an alkalinization of the vacuole was observed (from pH 5.7 to 6.1, as visualized by the shift of the resonance peak of vacuolar Pi, from 0.3 to 0.45 ppm),
time of sucrose starvation:
120 h

P-Cho
P-EA
~

Glc-6-P

Figure 1. Representative in vitro $^{31}$P-NMR spectra (perchloric acid extracts, expanded scale from 0 to 2.5 ppm and 3.5 to 5 ppm) of sucrose-supplied sycamore cells and cells starved for various times (12-120 h). Cells harvested from the culture medium were rinsed three times by successive resuspension in fresh culture medium devoid of sucrose and incubated at zero time in sucrose-free culture medium. The spectra recorded at 20°C are the result of 1,024 transients (1 h). Peak assignments: Fru-6-P, fructose 6-P; Glc-6-P, glucose 6-P; Man-6-P, mannose 6-P; GPC, glycerylphosphorylglycerol; GPE, glycerylphosphorylethanolamine; GPI, glycerylphosphorylinositol; PGA, 3-phosphoglycerate; P-EA, phosphorylethanolamine; P-Cho, phosphorylcholine.

which could result from an accumulation of ammonium deriving from protein catabolism.

Second, we observed a transient increase in the amount of sn-glycerol 3-P (at 4.4 ppm in extracts), glycerylphosphorylglycerol (GPC) (at 1.2 ppm in extracts), GPE (at 0.7 ppm in extracts), and GPC (at -0.1 ppm in extracts). Phosphodiester resonance peaks, clearly characterized in cell perchloric extracts at pH 7.5 (Fig. 1), were partially masked by the large vacuolar ß-Pi peak in the in vivo spectra (Fig. 2). However, since the chemical shifts of the phosphate group of phosphodiesters are not sensitive to the pH (physiological range), it is not possible to determine in which compartment (vacuole or cytoplasm) they accumulate.

Third, P-Cho (at 3.5 ppm in extracts) accumulated steadily, according to previous reports (Roby et al., 1987). In addition, an accumulation of phosphorylethanolamine (P-EA) (at 3.95 ppm in extracts) was also characterized. Titration curves plotting chemical shift vs pH for P-Cho and P-EA in crude cell extract (Fig. 2, inset) show that the position of phosphate group in starved cells corresponded to P-Cho and P-EA at pH > 7.2. This indicates that both phosphorylated compounds accumulated in the cytoplasmic compartment. With our experimental conditions and the assumption of a vacuolar volume to cytoplasmic volume ratio reaching 10 (the ratio in control cells is ~5-6, and it raises during starvation since the cytoplasmic volume markedly decreased owing to autophagy), the cytoplasmic P-Cho and P-EA concentrations attained after 48 h of sucrose starvation were 10-12 mM and 4-5 mM, respectively.

Fourth, phytate declined during the course of sucrose starvation, suggesting that it was metabolized (phytate appeared as a complex quadruple resonance clearly visible insofar as the perchloric acid extract contains an excess of 1,2-cyclohexylenedinitrilotetraacetic acid that chelates Mg$^{2+}$ engaged with it).

Fig. 3 shows the time-course evolution of P-Cho, P-EA, sn-glycerol-3-P, GPC, and GPE in sycamore cells subjected to sucrose starvation for up to 120 h. These compounds were quantified from the cell extract $^{31}$P-NMR spectra; the peak intensity of each phosphate ester (or diester) resonance was calibrated by adding known amounts of external phosphate ester (or diester) (see Materials and Methods). The evolution of phosphatidylethanolamine and phosphatidylethanolamine is also given in Fig. 3 (these phospholipids were quantified from biochemical analysis; see Materials and Methods).

The accumulation of P-Cho and P-EA parallels the decline of the corresponding phospholipids. Phosphodiesters (GPC and GPE) and sn-glycerol-3-P transiently accumulated during the hours preceding the steady accumulation
Figure 3. Time-course evolution of the most abundant phospholipids (phosphatidylcholine and phosphatidylethanolamine) and their degradation products in sycamore cells subjected to sucrose starvation for up to 120 h. Cells harvested from the culture medium were rinsed three times by successive resuspension in fresh culture medium devoid of sucrose and incubated at zero time into flasks containing sucrose-free culture medium. At each time, two sets of cells were harvested. In the first one, the contents of phospholipid degradation products were determined by 31P-NMR as described in Materials and Methods (see Fig. 1). The contents of phospholipids were determined biochemically (see Materials and Methods) from the second set of cells.

of P-Cho and P-EA in the cytoplasmic compartment. The time course also shows that the cytoplasmic P-EA that had built up in the absence of sucrose in the culture medium decreased slowly after a very long period of sucrose starvation. In contrast, under the same conditions, the final cytoplasmic P-Cho concentration remained remarkably stable up to the cell death that occurred ~7–8 d after the beginning of sucrose starvation. This result strongly suggests that P-Cho, unlike P-EA, was not further metabolized. Fig. 3 also indicates that the total amount of P-Cho that appeared in the cytoplasmic compartment corresponded to the total amount of phosphatidylcholine that disappeared within the same period of time. For example, after 4 d of sucrose starvation, the total amount of P-Cho present in intact sycamore cells was ~1.1 μmol per g of cell wet weight, and the total amount of phosphatidylcholine that had been hydrolyzed was ~1.2 μmol per g of cell wet weight. Furthermore, galactolipids, the lipids specific to the plastid envelope (Douce and Joyard, 1980b), were severely affected by carbohydrate starvation: they dropped from 152 μg per g fresh weight in control cells (i.e., sucrose-supplemented cells) to 82 μg per g fresh weight in cells starved for 4 d (Table 1). On the contrary, Table 1 indicates that sterols, which are mainly associated with plasmalemma and tonoplast membranes (Hartmann and Benveniste, 1987), were not significantly affected by sucrose starvation.

Taken together, these biochemical observations strongly suggest that incubation of sycamore cells in sucrose-free culture medium led to a marked loss of the intracellular membrane systems including plastids. Journet et al. (1986) also came to the same suggestion, as they demonstrated that the number of mitochondria per cell progressively declined during the course of sucrose starvation. To confirm this proposition, we have examined by EM the effects of sucrose starvation on the major cell membrane systems in sycamore cells.

**Ultrastructural Characterization of Autophagy**

**Effects of Sucrose Starvation on Cell Organelles.** The control cells (i.e., sucrose-supplemented cells) had a few large vacuoles that contained intravacuolar membrane remnants (Fig. 4 a) (Marty, 1978). Besides the components of the endomembrane system (secretory system), including the RER and dictyosomes and associated vesicles, plastids, mitochondria, and peroxisomes were observed in the cytoplasm. Voluminous plastids were the most conspicuous organelles because they contained big starch grains. When sucrose was omitted from the nutrient medium for 4 d, all of the starch grains sequestered in the stroma of plastids disappeared, and amyloplasts were converted into leucoplasts that displayed a dense matrix and a few internal membranes (Fig. 4 b). We inferred, therefore, that the starch formed in amyloplasts was broken down during the course of sucrose starvation, according to previous reports (Journet et al., 1986; Chan et al., 1994). In addition, mitochondria from cells deprived of sucrose for 4 d showed a darkened matrix as well as extensively developed cristae (Fig. 4 b2), as observed in starved maize root tips (Couée et al., 1992). A comparison between cells normally supplied with sucrose and cells deprived of the carbon source showed that the total number of mitochondria per cell decreased sharply with time, while the number of peroxisomes containing a catalase crystal increased (Fig. 5 c). This observation is consistent with an increased fatty acid oxidation in peroxisomes (Gerhardt, 1993). After 6 d of sucrose starvation, the total number of leucoplasts, mitochondria, and peroxisomes had decreased dramatically (to <20%), although the cells were still viable (not shown). Notably, after a long period of sucrose starvation, all of the cell organelles were localized almost exclusively in the immediate vicinity of the nucleus (Fig. 4 b), and no organelles were usually detected in the thin layer of cytoplasm squeezed between the cell wall and the large central vacuole (Fig. 4 b and b1). The cell size remained almost unchanged, but the cytoplasm appressed along the cell wall was considerably thinned. Cell death occurred usually

### Table I. Comparison of the Protein, Phospholipid, Galactolipid, and Sterol Contents in Sycamore Cells Supplemented with Sucrose or Starved in Carbohydrates for 4 d

|                | Proteins mg/g FW | Phospholipids μg/g FW | Galactolipids μg/g FW | Sterols μg/g FW |
|----------------|------------------|----------------------|----------------------|----------------|
| Control        | 17               | 2.1                  | 152                  | 125            |
| Sucrose starved| 9                | 1.1                  | 82                   | 121            |

Extractions and determinations were carried out according to the procedures described in Materials and Methods. The values, expressed in mg/g or μg/g fresh weight, are from a representative experiment.
Formation of Autophagic Vacuoles Induced by Sucrose Starvation. The electron micrographs in Fig. 5 illustrate the presence of autophagic vacuoles in the cytoplasmic compartment of cells that were deprived of sucrose for 14 h (Fig. 5 b) and 2 d (Fig. 5, a and c–e). Interestingly, these vacuoles were polymorphic, and on the basis of their content, boundary, and final fate, two pools could be clearly distinguished.

The largest pool consisted of double membrane-bound vacuoles that contained a fine fibrillar material dispersed in a clear sap (Fig. 5, a and b). Abundant membrane remnants were seen inside the vacuole, and figures indicative of complete sequences of autophagy were readily observed in the cytoplasm of cells deprived of sucrose for only 14 h (see, for instance, Fig. 5 b). These vacuoles were most frequently observed in the cells when all the endogenous reserves of carbohydrates (starch and sucrose) had been consumed (i.e., 1–2 d after the beginning of sucrose starvation) (Fig. 5 a). In addition, vesicles with the same average diameters and similar contents, but single membrane bound, were observed inside the vacuole system (Fig. 5 a). These figures suggest that autophagic vacuoles were formed in the peripheral cytoplasm during sucrose starvation and were eventually expelled into the large central vacuole for final disposal. Membrane rearrangements must have occurred when the double membrane-bound vacuoles were released into the central vacuole as single membrane-bound vesicles. In fact, small vacuoles newly made in the cytoplasm began to protrude in the central vacuole before they eventually dropped in it. In the protruding area, apparent membrane breaks were frequently seen (Fig. 5 d, arrowheads). However, such membrane fragmentation is usually cautiously interpreted as artifact (see Bowers and Maser, 1988; Hayat, 1989). In this regard, a fragmentation of the vacuole should lead to an acidification of cytoplasm and a progressive convergence of pH of both compartments. 31P-NMR studies indicated that this did not occur, since the cytoplasmic pH was not modified after 48 h of sucrose starvation (see Fig. 2) and thereafter until cell death (data not shown). The membrane events occurring where the outermost membrane of the small autophagic vacuole and the tonoplast of the central vacuole are pressed closely together are likely highly dynamic and complex (Fig. 5, d and e). Additional work using preservation strategies more refined than the usual chemical fixation is therefore needed for a detailed description of these fragile structures. Although the dynamics of the processes has to be better understood, these figures were interpreted as transient steps in the passage of the autophagic vacuoles from the cytoplasm to the central vacuole. The limiting membrane of the intravacuolar vesicles was the outermost membrane of the autophagic vacuoles previously found in the peripheral cytoplasm; its protoplasmic surface became exposed, therefore, to the aggressive vacuolar sap containing numerous lytic enzymes.

Vacuoles forming a second pool were also detected, although less frequently, in the cytoplasm of sycamore cells that were deprived of sucrose (Fig. 5 c). These vacuoles contained a variety of cellular structures at different stages of degradation including swollen organelles. Very often the cellular debris included sheets of membrane in multilamellar arrangements resembling the “myelinic figures” obtained after degradation of plastid membranes by phospholipase A2 (for review see Douce and Joyard, 1980b). No such vacuoles with cytoplasmic remnants were observed in the central vacuole of starved cells, and we still do not know whether these vacuoles were biogenetically and functionally related to the previous set of vacuoles described above.

These results are consistent with the massive breakdown of membrane lipids revealed by biochemical approaches. Cytoplasmic regions containing organelles are likely degraded in the autophagic vacuoles, which should involve many lytic enzymes. Interestingly, enzymatic activities involved in fatty acid β-oxidation (Dieuade et al., 1993) or proteolysis (James et al., 1993) have been shown to be induced by carbohydrate starvation, which is consistent with the use of amino acids and fatty acids as respiratory substrates in place of sugars. James et al. (1993) suggested that the induction of proteolytic activities in glucose-starved maize root tips is controlled by the level of sugars. This idea is supported by the fact that metabolic repression of transcription of many genes by sugars has been reported (for review see Sheen, 1994). To test if the induction of autophagy in sucrose-starved sycamore cells was controlled by intracellular sucrose and/or hexose phosphates below threshold concentrations, we used glycerol or pyruvate as a unique source of carbon. P-Cho, previously shown to reflect the extent of polar lipid degradation (see above), was used as a reliable marker for the autophagic process.

Effects of Glycerol or Pyruvate on Autophagy

In a previous paper (Aubert et al., 1994), we have reported that glycerol enters the cells by diffusion and that it

Figure 4. Representative sycamore cells, grown in the normal basic culture medium containing 50 mM sucrose (a) or cultivated for 4 d in sucrose-free culture medium (b). Cells harvested from the culture medium were fixed, embedded in epoxy resin, sectioned, and stained for EM as described in Materials and Methods. (a) Large globular vacuoles (V) are surrounded by the cytoplasm with numerous organelles. The plastids are the most conspicuous organelles because they contain large starch grains (S), but mitochondria (M), peroxisomes (P), RER, and Golgi profiles (G) as well as vesicles are also observed. CW, cell wall. (b) Nuclear region of a cell deprived of sucrose for 4 d. Plastids (PL), mitochondria (M), peroxisomes (P), and small globular vacuoles are almost exclusively localized around the nucleus (N), whereas everywhere else the cytoplasm remains very thin with only ribosomes and scarce RER profiles; CW, cell wall. (b1, inset) High magnification view of a region of cytoplasm containing only ribosomes, and occasional vesicle profiles squeezed between the plasma membrane (PM) and the tonoplast (T). (b2, inset) Example of mitochondrion displaying extensive cristae with a fairly complex topology in a dense matrix. Bars: (a and b) 1 mm; (b1 and b2) 100 nm.
can sustain respiration at normal rates for many weeks when used as the sole source of exogenous carbon in the culture medium of sycamore cells. Glycerol is first phosphorylated to sn-glycerol-3-P (Fig. 6), which accumulates in the cytoplasm and is further oxidized to fuel the glycolysis. Under these conditions, the recycling of triose phosphates back to hexose phosphates is prevented because the high concentrations of cytoplasmic sn-glycerol-3-P inhibit glucose-6-phosphate isomerase. Therefore, the synthesis of hexose phosphates and sugars is stopped as well as growth. Figs. 6 and 7 (●) show that the addition of 50 mM glycerol just as the very beginning of sucrose starvation prevents the accumulation of P-Cho, while cells are rapidly depleted in hexose phosphates (Fig. 6) and carbohydrate reserves (not shown). Under these conditions, cells cultivated with glycerol as a sole source of carbon can survive for weeks without any sign of polar lipid hydrolysis (no accumulation of P-Cho) or structural autophagy (absence of autophagic vacuoles). Conversely, we observed that the removal of the external glycerol (by extensive washing with carbohydrate-free medium) triggered a rapid accumulation of P-Cho, the biochemical marker of autophagy, almost without a lag phase (Fig. 7, ●). Concomitantly, respiration declined, and we verified that a rapid formation of autophagic vacuoles (similar to those shown in Fig. 5, a and b) occurred (not shown). On the other hand, when 10 mM glycerol was added back to the cells, the accumulation of P-Cho stopped almost immediately (Fig. 7, ●). We must point out the fact that the maximal rate of accumulation of P-Cho after removing glycerol was higher than that observed when control cells were deprived with sucrose (Fig. 7, ○). This probably corresponds to the fact that control cells are heterogeneous in terms of carbohydrate reserves. Thus, they are affected by autophagy after various times of starvation. On the contrary, cells cultivated with glycerol have no reserves and are synchronously affected by autophagy. One mechanism by which glycerol prevented autophagy could be the supply of respiratory substrates to mitochondria via glycolysis. To support this hypothesis, similar experiments were carried out using pyruvate, one of the main mitochondrial substrates directly deriving from glycolysis, as the unique source of carbon for sycamore cells. As for glycerol, the addition of 1 mM (higher concentrations are toxic to the cells, due to acidification of the cytoplasm [Kurkdjian and Guern, 1989]) pyruvate at the very beginning of sucrose starvation prevented the accumulation of P-Cho (Fig. 7, ●). Pyruvate entered the cells, probably under its undisassociated form, and at equilibrium the cell concentration was 8–10 mM when pyruvate was applied at the external concentration of 1 mM at pH 6.5 (13C-NMR analysis, result not shown). Cells were rapidly depleted in sugars and hexose phosphates (Fig. 6), indicating that the reflux from pyruvate back to hexose phosphates did not occur. Under these conditions, P-Cho did not accumulate and no autophagic vacuole was observed. In contrast, when pyruvate was removed from the culture medium, P-Cho rapidly accumulated without a lag phase (Fig. 7, ●). We verified that cell respiration rapidly declined and that autophagic vacuoles were formed. This evolution was stopped by the addition of either glycerol or pyruvate (Fig. 7, ○).

**Discussion**

Our results illustrate the high structural and metabolic plasticity of higher plant cells, which can cope with >6 d without a supply of carbohydrates.

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**Figure 5.** Autophagic vacuoles in sycamore cells grown for 14 h (b) and 2 d (a and c-e) in sucrose-free culture medium. Cells harvested from the sucrose-free culture medium were fixed, embedded in epoxy resin, sectioned, and stained for EM as described in Materials and Methods. (a) Nuclear region of a sucrose-starved cell in which globular vacuoles (V*, ~3–5 μm in diameter) formed in the peripheral cytoplasm are being released in the central large vacuole (V). (b) Double membrane-bound vacuolar inclusion in the cytoplasm of a cell deprived of sucrose for 14 h. The inner (arrowheads) and outermost (arrows) membranes initially act in the sequestration process that takes place early in autophagy. (c) Single membrane-bound vacuolar inclusion in the nuclear region of a cell deprived of sucrose for 2 d. The intravacuolar content is filled with membrane bilayers in multilamellar arrangements and with other cytoplasmic debris; same type of vacuolar inclusions with swollen organites as well as vesicles and globules bathing in a fine granular sap were found in the peripheral cytoplasm of cells grown for 8 d in a culture medium devoid of sucrose; P, peroxisome. (d) Vacuoles with a clear content are protruding into the central vacuole; M, mitochondrion. (e) High magnification view of an area where the membrane boundary of the autophagic vacuole and the tonoplast are pressed closely together (similar to that indicated by brackets on Fig. 5 d); CW, cell wall. Bars: (a and d) 1 mm; (b and c) 500 nm; (e) 250 nm.

**Figure 6.** Representative in vitro 31P-NMR spectra (perchloric extracts, expanded scale from 0 to 5 ppm) of sycamore cells cultivated with sucrose, glycerol, pyruvate, or without any carbon source. Cells harvested from the culture medium were rinsed three times by successive resuspension in fresh culture medium devoid of sucrose and incubated at zero time into flasks containing sucrose-free culture medium supplemented or not with glycerol or pyruvate. A, control cells; B, cells cultivated without any carbon source for 3 d; C, cells cultivated with 50 mM glycerol as the sole source of carbon for 3 d; D, cells cultivated with 1 mM pyruvate as the sole source of carbon for 3 d. The spectra recorded at 20°C are the result of 4,048 transients (2 h). Peak assignments are the same as in Fig. 1.
31P-NMR spectra from cell extracts, according to the procedures described in Materials and Methods. Arrows indicate the time when glycerol or pyruvate was removed (by extensive washing with sucrose-free culture medium) or added again. At each time point, P-Cho was determined and quantified from 31P-NMR spectra from cell extracts, according to the procedures described in Materials and Methods.

**Structural Evidence for Autophagy**

We showed that after a long period of sucrose starvation, sycamore cells undergo a reversible autophagy, characterized by the formation in the cytoplasmic compartment of numerous double membrane–bound vesicles analogous to autophagosomes described in various mammalian cells (Dunn, 1990a), yeast (Baba et al., 1994), and differentiating meristematic plant cells (Marty, 1978). In this latter case, cytochemical studies at conventional and high voltage EM have shown that the cavity and membrane boundaries of the autophagosomes derive from a post-Golgi prevacuolar compartment (Marty, 1978). Conversely, immunocytochemical studies have shown that autophagosomes in animal cells originate from ribosome-free regions of the RER (Dunn, 1990a). Experiments are undertaken to specify the origin of autophagosomes in carbohydrate-starved sycamore cells. We further observed that autophagosomes were expelled to the central vacuole reservoir, a compartment specific for plant and yeast cells, thought to be functionally equivalent to animal lysosomes (Matile, 1975). The arguments in favor of this pattern are the following: (a) autophagosomes are the first type of autophagic vesicles observed in starved cells from 14 h of starvation (Fig. 5 b); (b) single membrane–bound vesicles, similar in size and in aspect to autophagosomes, were observed within the central vacuole; and (c) transient steps in the passage of the autophagosomes to the central vacuole were observed, involving interaction between the tonoplast and the membranes of the autophagic vacuole (Fig. 5, d and e). This pattern resembles the autophagic sequences characterized in yeast shifted to nutrient starvation media (Baba et al., 1994). Indeed, these authors demonstrated that the “autophagic bodies” (single membrane vesicles containing cytosol) reported in the central vacuole of starved yeasts (Takeshige et al., 1992) originated from autophagosomes formed in the cytoplasm. In addition, the autophagic process we describe in this paper is reminiscent of the autophagy leading to the formation of vacuoles in differentiating meristematic cells (Marty, 1978; Marty et al., 1980). On the other hand, it is different from the autophagic process involving the invagination of the tonoplast and the subsequent engulfment of cytoplasmic fragments into the cavity of the preexisting vacuole (Wittenbach et al., 1982; Van der Wilden et al., 1980; Melroy and Herman, 1991).

The question of the regulation of membrane polar lipid hydrolysis during the course of autophagy remains unclear, and in particular, the problem of fatty acid movement between the autophagic vacuole and the remaining cytoplasm where they are oxidized via β-oxidation either in the peroxisomes (Gerhardt, 1993) or in the mitochondria (Thomas et al., 1988; Dieuaide et al., 1993). Since the half-life of autophagic vesicles in mammalian cells has been estimated to be ~10 min, indicating that their contents are quickly degraded (for review see Marzella and Glaumann, 1987), it may be possible that free fatty acids, which behave like strong detergents, accumulate transiently in the autophagic vacuoles, and then are released progressively to the cytoplasmic compartment for respiratory purposes.

These structural observations shed new light on the regression of the mitochondrial system previously described in sucrose-deprived sycamore cells using biochemical techniques (Journet et al., 1986).

**P-Cho as a Reliable Biochemical Marker of Autophagy in Carbohydrate-starved Cells**

The appearance of autophagic vacuoles paralleled the breakdown of membrane lipids. A general decline of fatty acids during the course of carbohydrate starvation had been described previously (Dorne et al., 1987). Here we report that sterols were not significantly affected by carbohydrate starvation. As they are mainly associated with plasmalemma and tonoplast (Hartmann and Benveniste, 1987), we inferred that these membrane systems were spared by autophagy. This observation is consistent with the increase of the volume of the main central vacuole during sucrose deprivation, and with the major role of plasmalemma as a requirement for cell integrity (which is maintained up to 7–8 d). In contrast with sterols, we observed a marked decrease of galactolipids, which are highly restricted to the plastid in plant cells (Douce and Joyard, 1980b). This result strongly suggested that plastids were degraded in sugar-starved cells. It is consistent with the fact that some autophagic vacuoles observed during sucrose starvation contained swollen organelles like plastids. Interestingly, vacuoles containing plastids have been reported in wheat leaves (Wittenbach et al., 1982) during senescence, a step of development that probably involves autophagic activity (Matile, 1987). Furthermore, we described the decline of phosphatidylethanolamine and phosphatidylcholine, the two main membrane phospholipids.

![Figure 7](image-url)
of sycamore cells. Fig. 3 indicated that the accumulation of P-Cho, first observed by Dorne et al. (1987), derived from phosphatidylcholine degradation and not from de novo synthesis and absence of incorporation into membrane systems of nongrowing starved cells. The results presented in this paper strongly suggest that hydrolysis of membrane polar lipids occurred in several distinct steps. Indeed, a careful analysis of Fig. 3 demonstrates that phosphodiesterases, which accumulated transiently, appeared before the steady accumulation of P-Cho and P-EA. It is very likely that after sequestration of the membrane systems within the autophagic vacuoles, net lipid breakdown commences. GPC (and GPE) probably derive from the attack of membrane polar lipids by nonspecific acyl hydrolases (Galliard, 1980; Huang, 1993). Hydrolysis of GPC could lead to either glycerol and P-Cho, or to sn-glycerol-3-P and choline. Since the cytoplasmic compartment contains powerful glycerol kinase (EC 2.7.1.30) (Hippman and Heinz, 1976; Aubert et al., 1994) and choline kinase (EC 2.7.1.32) (Weretilnyk and Summers, 1992; Bligny et al., 1989), glycerol and choline thus formed are immediately phosphorylated, leading to the formation of sn-glycerol-3-P and P-Cho. Experiments are undertaken in our laboratory to discriminate between the two possibilities. However, it does not preclude the direct hydrolysis of polar lipids by phospholipase D, leading to the formation of choline and phosphatidic acid (Wang, 1993). This last hypothesis is most unlikely because we did not observe a transient accumulation of phosphatidic acid during the course of sucrose starvation.

It is surprising that even after a very long period of sucrose starvation, P-Cho that accumulated in the cytoplasmic compartment exhibits a remarkable metabolic inertness. Indeed, according to Martin and Tolbert (1983), P-Cho is a particularly stable molecule: it is resistant to strong acid hydrolysis and is hardly hydrolyzed by intracellular acid phosphatases. Only when growth is reinitiated, i.e., when sucrose is added to the medium of starved cells, will P-Cho decline at the rate of its use for membrane lipid synthesis (Roby et al., 1987). Consequently, the total amount of P-Cho present within the cell reflects the extent of intracellular membrane degradation during the course of sucrose starvation. In addition, the time course of accumulation of P-Cho perfectly matches the decline of cell respiration (see Journet et al., 1986). Interestingly, we also observed that accumulation of P-Cho occurred in cell suspensions of the white campion Silene alba and the weed monocot Echinochloa column during carbohydrate starvation (manuscript in preparation). Similar biochemical investigations would be necessary to know if our results can be generalized to the other situations of autophagy in plants (differentiating cells, senescing leaves, or cotyledons of germinating seedlings). Likewise, it would be of particular interest to study the degradation of membrane lipids and to see if deacylation products such as GPC and P-Cho accumulate in animal and yeast starved cells. Indeed, most biochemical work on autophagy in these systems is related to proteolysis (Grinde, 1985).

P-EA also accumulated during carbohydrate starvation and is an indicator of autophagy. However, in contrast with P-Cho, it can be metabolized (its fate is unknown) after a prolonged time of starvation. Other indicators of autophagy have been proposed, such as the amino acid asparagine (Génix et al., 1990). Indeed, carbohydrate starvation leads to the accumulation of free amino acids, with asparagine being prevalent since it is a detoxification compound acting in nitrogen storage. However, the accumulations of asparagine, as well as other amino acids, are transient and decline after very long periods of starvation (Brouquisse et al., 1992). The same holds true for the induction of the enzymes involved in protein hydrolysis and asparagine metabolism (James et al., 1993). In contrast, owing to its remarkable metabolic inertness, P-Cho appears as a reliable marker of autophagy in plant cells reflecting the extent of this process, which depends on the time of starvation and on other factors such as the incubation temperature of the cells. Indeed, preliminary experiments carried out in our laboratory show that the accumulation of P-Cho is markedly slowed down when cells are starved at 10°C, corresponding to the fact that metabolism is diminished. The use of P-Cho as a marker of autophagy opens the way to screen for mutants that are autophagy deficient. Here, we used this marker in combination with other indicators (decline of respiration, autophagic vacuoles) to investigate the factors involved in the control of autophagy.

Control of Autophagy by the Supply of Mitochondria with Respiratory Substrates

James et al. (1993) suggested that the induction of proteolytic activities in glucose-starved maize root tips is controlled by the level of sugars at any moment during the starvation period. This idea is supported by the fact that the transcription of many genes has been shown to be regulated by sugars (for review see Sheen, 1994). For instance, the expression of α-amylase genes (Chen et al., 1995) and genes involved in glyoxylate cycle (Graham et al., 1994) is stimulated by carbohydrate starvation and repressed by sugars. Graham et al. (1994) suggested that the signal giving rise to a change in glyoxylate cycle gene expression originates from the intracellular concentration of hexose sugars or the flux of hexose sugars into glycolysis. Additionally, carbon catabolite repression is well known in bacteria (Ullmann, 1985) and in yeasts (Gancedo, 1992). In yeast, hexose phosphorylation by hexokinases seems to be involved in this phenomenon (Rose et al., 1991). We could thus hypothesize that the autophagic process (including the induction of lytic enzymes) might be controlled by the level of sugars or hexose phosphates, which rapidly decline during carbohydrate starvation (see Journet et al., 1986).

Our results show that glycerol, when used as the sole source of carbon, could prevent the accumulation of P-Cho (Figs. 6 and 7). No sign of autophagy (at biochemical and structural levels) was observed even after weeks, while cells were depleted of sugars and hexose phosphates. These observations suggest that autophagy is not engaged as long as mitochondria are provided with respiratory substrates deriving from the glycolytic pathway. However, we cannot exclude a direct repression by glycerol (or sn-glycerol-3-P) of the machinery involved in the autophagic process. Interestingly, Sheen (1990) observed that glycerol repressed the activity of two photosynthetic genes with similar effi-
Pyruvate is the main mitochondrial substrate derived from glycolysis, but P-Cho did not accumulate and autophagy did not occur. Whether the repression originates from glycerol (or sn-glycerol-3-P) itself or from the fact that glycerol fueled mitochondria with respiratory substrates via glycolysis. The results obtained with pyruvate as the sole source of carbon argue in favor of the latter hypothesis. Indeed, we observed that pyruvate entered the cells, as previously reported in maize cells by Ashworth et al. (1987), and sustained normal rates of respiration. As with glycerol, cells were rapidly depleted in sugars and hexasse phosphates, but P-Cho did not accumulate and autophagy did not occur. Little is known about the direct effects of pyruvate on gene expression. It has been reported that one photosynthetic gene (encoding the maize C4 pyruvate phosphodi-kinase) was not affected by pyruvate, while it was stongly repressed by sugars, glycerol, or acetate (Sheen, 1990). As pyruvate is the main mitochondrial substrate derived from glycolysis, we suggest that pyruvate prevented autophagy by supplying the mitochondrial electron transport chain and phosphorylating system. In support of this suggestion, we observed that the removal of pyruvate from the culture medium led to the rapid accumulation of P-Chol without a lag phase (Fig. 7). This induction of autophagy was stopped by an addition of glycerol or pyruvate. These results lead us to conclude that the signal(s) giving rise to the autophagic process originate(s) from the decline of the supply of respiratory substrates to the mitochondria and not simply from the decrease of sugars and sugar phosphates to threshold concentrations.

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