Effect of sucrose starvation on sycamore (*Acer pseudoplatanus*) cell carbohydrate and P_i status

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(Received 2 August 1984/Accepted 12 November 1984)

The mobilization of stored carbohydrates during sucrose starvation was studied with sycamore (*Acer pseudoplatanus*) cells. When sucrose was omitted from the nutrient medium, the intracellular sucrose pool decreased rapidly during the first hours of the experiment, whereas the starch content remained practically unchanged. After 10 h of sucrose starvation, starch hydrolysis replaced sucrose breakdown. From this moment, the phosphate-ester pool and respiration rate decreased with time. Conversely, the intracellular P_i concentration increased. ^{31}P n.m.r. of intact sycamore cells indicated that, under these conditions, most of the P_i accumulated in the vacuole. These results strongly suggest that starch breakdown, in contrast with sucrose hydrolysis, is not rapid enough to maintain a high cellular metabolism.

An understanding of how higher plants regulate the mobilization of stored carbohydrates is of great agronomic importance. It is generally accepted that sucrose is the principal form in which carbohydrate is transported from photosynthetic tissues to organs where starch is deposited (Giaquinta, 1980; Preiss, 1982), and there is a correlation between the rate of starch synthesis and the level of soluble carbohydrates (Jenner, 1970). In recent years much progress has been made towards the elucidation of the biochemical aspects of starch synthesis and breakdown. Starch synthesis in leaf cells is mainly controlled by ADP-glucose pyrophosphorylase, and there is good evidence that phosphorolysis and glycolysis constitute a major pathway of starch breakdown in chloroplasts (Preiss, 1982). These activities are regulated largely by the intracellular level of P_i and triose phosphate (Herold *et al.*, 1976; Heldt *et al.*, 1977; Kaiser & Bassham, 1979; Stitt & Heldt, 1981; Preiss, 1982; Edwards & Walker, 1983).

Compared with chloroplasts, very little is known about starch metabolism in amyloplasts (Macdonald & ap Rees, 1983). With the aim of further clarifying the mechanism of starch metabolism in non-green cells, the present paper reports a detailed account of the simultaneous measurement of sucrose, starch, cytoplasmic P_i , vacuolar P_i ,

phosphate esters and O_2 consumption of sycamore (*Acer pseudoplatanus*) cells when the cells are grown in a medium devoid of sucrose.

Experimental procedure

Plant material

The strain of sycamore (*Acer pseudoplatanus* L.) used in the present study was generously provided by J. Guern (Plant Cellular Physiology, Gif-sur-Yvette, France). The basic nutrient medium was prepared as described by Bligny (1977), and cell suspensions were maintained in exponential growth by frequent subcultures. The culture medium was kept at a volume of 0.2 litre and stirred continuously with a pair of impellers at 60 rev./min. Under our culture conditions the cell number increased exponentially without a lag phase.

^{31}P n.m.r. of cells

With a spectrometer (WM 200, Bruker) operating at 81.01 MHz for ^{31}P and the pulsed-Fourier-transform technique, it is possible to resolve and assign peaks to various intracellular pools of P_i known to be present in the plant cells (Roberts *et al.*, 1980; Foyer *et al.*, 1982; Kime *et al.*, 1982; Martin *et al.*, 1982; Waterton *et al.*, 1983). By this method one may discriminate P_i present in the

vacuole (acidic compartment) from that present in the slightly alkaline cytoplasm (soluble phase of the cytoplasm plus all its organelles). The method, which is non-invasive, relies on the fact that the signals obtained from P_i in different states of ionization occur at different frequencies.

The preparation of cells suspensions to record ^{31}P n.m.r. spectra is fully described elsewhere (Martin *et al.*, 1982). Cells (4g wet wt.), carefully washed with P_i -free culture medium, were introduced into a 25mm-diameter tube with 20ml of P_i -free buffered culture medium, pH 6.5. The cell suspension was bubbled with air. Spectra were recorded after 900 up to 25000 accumulations with a repetition time of 1.20s. We have verified that these experimental conditions allowed a full relaxation of both vacuolar and cytoplasmic P_i between each scan. The position of the P_i peak was estimated in 5mM-KOH/ K_2HPO_4 buffers at given pH values. ^{31}P n.m.r. provides not only a non-destructive mean of observing free P_i directly, but also allows an approximation of the relative concentration of P_i present in each major intracellular compartment, i.e. the cytoplasm and the vacuole. By using KH_2PO_4 /KOH-buffered solutions at different pH values between 4 and 9, we have observed in several separate experiments that there exists a good linear relationship between the P_i peak intensity (areas under peaks) and P_i concentration. The inaccuracies in determining intracellular P_i concentrations are only limited by the uncertainty in determining the area of the wide intracellular P_i resonance peaks. Areas were compared by including a reference capillary containing 70mM-methylene diphosphonate (pH 8.9 in 30mM-Tris). Assignments of peaks in cell ^{31}P n.m.r. spectra (Kime *et al.*, 1982) are given in the legend to Fig. 2. Spectra were accumulated with the samples at a temperature of 295 ± 1 K.

Measurement of the phosphate pool

Cell samples (200mg wet wt.) were removed, strained on a glass-fibre filter [15s, pressure of suction 60kPa (0.6bar)], and rapidly rinsed three times with 10ml portions of distilled water.

For measurement of total P_i , cells were dropped immediately into 3ml of 10% (v/v) $HClO_4$ and left at 0°C for 15min. Each sample was then centrifuged for 10min at 20000g. Portions of the supernatant were removed and P_i was directly measured by a phosphomolybdic acid method (Taussky & Shorr, 1953). Total acid-soluble phosphorus (phosphate esters) was measured in like manner after digestion of a 200 μ l portion of $HClO_4$ extract in 50 μ l of 5M- H_2SO_4 at 180°C in Kjeldahl flasks until colourless. Phosphate ester was calculated as the difference between the total acid-soluble P and P_i .

For glucose 6-phosphate determination, $HClO_4$ extracts were neutralized by 5M-KOH (up to pH 5.5) and centrifuged for 10min at 20000g to remove $KClO_4$. Glucose 6-phosphate was then determined with glucose-6-phosphate dehydrogenase (EC 1.1.1.49) as described by Lang & Michal (1974). We have verified that hydrolysis of glucose 6-phosphate during killing and extractions of cells did not occur.

For ATP and ADP determinations, cells were very quickly frozen in diethyl ether at 143K to avoid ATP destruction by ATPase, then fixed with 2ml of 5% (v/v) $HClO_4$. $HClO_4$ extracts were neutralized by 5M-KOH and centrifuged for 10min at 20000g to remove $KClO_4$. ATP was determined with glucose-6-phosphate dehydrogenase and hexokinase (EC 2.7.1.1) by the method of Lamprecht & Trantschold (1974); ADP was determined with pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) by the method of Jaworek *et al.* (1974). The absolute concentration of cytoplasmic ATP was also determined by comparing areas of peaks in cell n.m.r. spectra with peak areas of standard ATP solutions.

Measurement of the sucrose and starch pool

Cell samples (200mg wet wt.) were removed, strained on a glass-fibre filter (15s, pressure of suction 60kPa) and rapidly rinsed three times with 10ml portions of distilled water. Cells were rapidly dropped into 3ml of ice-cold 0.5M-NaOH and left at 0°C for 15min. Portions (1ml) were removed for starch determination, vigorously stirred in a Potter-Elvehjem homogenizer to disrupt cells and plastids, then neutralized with 10M-HCl. Portions (500 μ l) were then incubated for 1h at 35°C with 500 μ l of amyloglucosidase (EC 3.2.1.3) (Sigma) (1mg/ml) in sodium acetate, pH 4.6. Glucose from starch hydrolysis was determined with glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and hexokinase (EC 2.7.1.1) as described by Bergmeyer *et al.* (1974).

For sucrose determination, NaOH extracts were centrifuged for 10min at 20000g and the supernatant was neutralized with 10M-HCl. Portions (200 μ l) were then incubated 1h at 25°C with 200 μ l of invertase (EC 3.2.1.26) (Sigma), 5mg/ml in sodium acetate pH 4.6. Glucose and fructose from sucrose hydrolysis were determined with glucose-6-phosphate dehydrogenase (EC 1.1.1.49), hexokinase (EC 2.7.1.1) and phosphoglucose isomerase (EC 5.3.1.9) as described by Bergmeyer *et al.* (1974) and Bernt & Bergmeyer (1974).

Measurement of O_2 consumption

Sycamore cell respiration was measured at 25°C in their culture medium. O_2 uptake was measured with a Clark-type oxygen-electrode system pur-

chased from Hansatech, King's Lynn, Norfolk, U.K. (Delieu & Walker, 1972). A value of 240 nmol of O_2 /ml was used for the solubility of O_2 at 25°C.

Results

Effect of sucrose starvation on carbohydrate and P_i pools

In view of the large intracellular sucrose concentration (80 ± 10 mM) and starch content (65 ± 10 mM, expressed as glucose) we decided to determine the fate of both sucrose and starch when cells were grown in a medium devoid of sucrose. Experiments were therefore designed to decrease the endogenous carbohydrate pool. When sucrose was omitted from the nutrient medium, the endogenous sucrose decreased to 30% of the control value within 14 h. We calculate from Fig. 1 that the rate of net breakdown of sucrose during the first 8 h was 5 μ mol of sucrose/h per g fresh weight. During this time the starch pool remained practically constant (Fig. 1a). Separate control experiments (results not shown), in which cells were grown in a medium containing [^{14}C]sucrose and then transferred to a sucrose-free medium,

indicated that very little sucrose efflux occurred. It is very likely that sucrose molecules liberated to the external medium were rapidly re-absorbed. Starch hydrolysis only occurred after 10 h of sucrose starvation, at which time the internal sucrose concentration was low (Fig. 1a).

After 10 h of sucrose starvation, the P_i concentration increased considerably with time (Fig. 1b). The simultaneous decrease of the phosphate-ester concentration (Fig. 1b) strongly suggests that P_i molecules that accumulate in the cells were liberated from the phosphate esters. Under these conditions, the glucose 6-phosphate pool (Fig. 1b) decreased in parallel with the phosphate-ester pool. It is noteworthy that the lag phase observed for P_i or phosphate-ester evolution was comparable with that observed for starch hydrolysis. However, the P_i concentrations of the two major intracellular compartments (i.e. the cytoplasm and the vacuole) are not identical and can fluctuate separately (Rébeillé *et al.*, 1982, 1983). In order to find out whether P_i plays a central role in starch breakdown, it is necessary to determine in which cellular compartment (i.e. the cytoplasm or the vacuole) P_i accumulates during sucrose starvation.

Fig. 1. Effect of sucrose starvation of sycamore cells on intracellular sucrose, starch, P_i , phosphate-ester and glucose 6-phosphate concentrations

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 (20 mg wet wt./ml) in flasks containing sucrose-free culture medium. (a) Time course of changes in sucrose and starch content. The starch concentration is expressed as nmol of glucose/mg wet wt. (b) Time course of changes in phosphate-ester, P_i and glucose 6-phosphate concentrations.

Experiments were therefore performed to determine the P_i concentration in the cytoplasmic compartment. Unfortunately this task is hampered by the presence of an acidic vacuole that occupies much of the cell volume, but ^{31}P n.m.r. can be used to discriminate between the vacuolar and cytoplasmic P_i pools.

^{31}P n.m.r. of cells

^{31}P n.m.r. spectra obtained from sycamore cells harvested at the end of exponential phase of growth (i.e., 5-day-old cells) showed two distinct peaks of intracellular P_i at approx. 2 and 0.25 p.p.m., equivalent to approx. pH 7.3 and 5.7 respectively (Fig. 2). These values reflect the presence of the vacuolar P_i pool at the acidic pH and the cytoplasmic P_i pool at the slightly alkaline pH (Roberts *et al.*, 1980; Martin *et al.*, 1982; Foyer *et al.*, 1982). The spectra of sycamore cells presented here are identical with those reported elsewhere (Martin *et al.*, 1982; Rébeillé *et al.*, 1983). As there exists a linear relationship between the P_i peak intensity and P_i concentration, we may assume that the ratio of vacuolar peak area to

cytoplasmic peak area is identical with the ratio of vacuolar P_i to cytoplasmic P_i . By considering the total intracellular P_i concentration and the ratio of vacuolar volume to cytoplasmic volume (roughly 9) (Bligny & Douce, 1976), we can estimate the cytoplasmic and the vacuolar P_i concentrations. For sycamore cells containing about 2 mM total P_i , the cytoplasmic and the vacuolar P_i concentrations were approx. 5 ± 1 and 1.65 ± 0.1 mM respectively (mean values obtained from 15 experiments). Interestingly, as the β -ATP peak intensity (the intracellular ATP concentration is approx. $0.5 \mu\text{mol/g}$ wet wt.) is equal to that of γ -ATP ($+\beta$ -ADP) peak (Fig. 2), we may assume that the absolute concentration of cytoplasmic ADP is not detectable by ^{31}P n.m.r.

When sucrose was omitted from the nutrient solution, the endogenous P_i pool increased within 30 h (Fig. 1). The ^{31}P n.m.r. spectra were obtained with intact cells to determine the P_i concentration in each intracellular compartment. Each spectrum was accumulated for 18 min (900 scans). Fig. 3 indicates that most of the P_i molecules liberated from phosphate-ester molecules were expelled into the vacuole, where they accumulated. In contrast, very few P_i molecules liberated from the phosphate-ester pool accumulated in the cytoplasm (Fig. 3). Since the decrease in phosphate esters is believed to be predominantly a cytoplasmic event, it would seem that, in sycamore cells, the P_i content of the cytoplasm is largely maintained at the expense of the P_i vacuolar pool.

Fig. 2. Representative ^{31}P n.m.r. spectra of sycamore cells harvested at the end of the exponential phase of growth

Cells (about 4 g wet wt.) were collected from the culture medium by filtration and rinsed three times by successive resuspensions in fresh culture medium buffered at pH 6 by 50 mM-Mops and devoid of P_i . Cells were suspended with 20 ml of the buffered standard medium without P_i , directly into the n.m.r. tube. The cell suspension was supplied with air during the experiment (see the Experimental procedure section). Spectra were obtained over a period of about 10 h. Peak assignments: 1, glucose 6-phosphate; 2, cytoplasmic P_i ; 3, vacuolar P_i ; 4 γ , γ -ATP ($+\beta$ -ADP); 4 α , α -ATP ($+\alpha$ -ADP); 5, 5', UDP-glucose; 6, nicotinamide-adenine nucleotides; 4 β , β -ATP. Shoulders to the right of 1 are unassigned. The position of the 4 β -ATP peak indicates that almost all the cytosolic ATP is complexed with Mg^{2+} .

Rate of O_2 consumption by sycamore cells

The rate of O_2 consumption by sycamore cells during the course of sucrose starvation remained constant during the first hours of experimentation (Fig. 4). After 18 h of sucrose starvation, the rate of O_2 consumption decreased with time to 50% of the control value within 40 h. It is noteworthy that the growth rate of sycamore cells also decreased considerably after 10 h of sucrose starvation (results not shown) and stopped completely after 20 h of sucrose starvation. Fig. 4 indicates that the uncoupled rate of O_2 consumption also decreased with time. Interestingly, the full rate of O_2 consumption by 70 h-sucrose-starved cells was recovered a few hours after the addition of sucrose (Fig. 4). This lag phase probably represents the time required to restart cell growth, which is an ATP consumer.

Discussion

The present results indicate that sycamore cells have two major pools of carbohydrates (sucrose and starch). It is obvious that starch is present in the stroma of amyloplasts. The high intracellular

Fig. 3. Representative experiment showing the effect of sucrose starvation on the vacuolar (vac-P_i) and cytoplasmic P_i (cyt-P_i) content of sycamore cells

Cells were harvested from the culture medium and rinsed three times by successive resuspension in fresh culture medium devoid of sucrose. Cells were incubated at zero time (30 mg wet wt./ml) in flasks containing sucrose-free culture medium. At various times, cells (about 4 g wet wt.) were harvested from the experimental medium, rinsed three times by successive resuspension in fresh culture medium devoid of sucrose and P_i , and suspended with 20 ml of the buffered culture medium (pH 6), without sucrose and P_i , directly into the n.m.r. tube. Total P_i (tot-P_i) was measured by the phosphomolybdic acid method of Taussky & Shorr (1953), and vacuolar and cytoplasmic P_i contents were calculated from n.m.r. spectra (see the Experimental procedure section).

sucrose concentration (80 $\mu\text{mol/g}$ wet wt.) and the small cytoplasmic-to-vacuolar-volume ratio indicate that most of the sucrose is probably stored in the vacuole. Indeed, according to Nishimura & Beevers (1978), a large proportion of the cellular sucrose is contained in the vacuole.

When sucrose is omitted from the nutrient medium, vacuolar sucrose is first consumed.

Fig. 4. Time course of sycamore cell respiration rate when cells are grown in a medium devoid of sucrose

Cells were harvested from the culture medium and rinsed three times by successive resuspension in fresh culture medium devoid of sucrose. Cells were incubated at zero time (20 mg wet wt./ml) into flasks containing sucrose-free culture medium. At each time, cells were harvested and O_2 -consumption rate was measured as described in the Experimental procedure section. ■, Normal respiration; ○, uncoupled respiration; the arrows indicate an addition of 50 mM sucrose.

During this time, the starch, P_i and phosphate-ester contents remain constant. The same holds true for the respiration rate. These results suggest that, at high vacuolar sucrose concentration, efflux of sucrose from the vacuole to the cytoplasm is sufficient to assure a high cellular metabolism. However, when a threshold of intracellular sucrose concentration is attained (i.e. after 10 h of sucrose starvation), the rate of sucrose efflux from the vacuole becomes a limiting factor for cytoplasmic glycolysis rate because the phosphate-ester pool decreases progressively with a parallel increase in intracellular P_i . Under these conditions, starch breakdown, which progressively replaces sucrose hydrolysis, is not rapid enough to maintain an optimum phosphate ester concentration, since growth rate decreases during this time. Most of the P_i formed at the expense of phosphate esters during the course of sucrose starvation is rapidly sequestered into vacuolar P_i pool. These results suggest, in good agreement with previous reports (Foyer *et al.*, 1982; Rébeillé *et al.*, 1983; Waterton *et al.*, 1983), that the vacuole compartment is able to 'buffer' the fluctuations of P_i concentrations occurring in the cytoplasm and therefore can exert a powerful control on cell metabolism. Such a situation should prevent rapid phosphorolysis of starch, which is promoted by P_i (Preiss & Levi, 1980; Edwards & Walker, 1983), in so far as phosphorylase(s) contribute(s) substantially to

starch breakdown in sycamore cells. However, we must bear in mind that there is still no information on the relative contributions of amylases (α -amylase, β -amylase) and α -glucan phosphorylase to starch breakdown in amyloplasts and in what form intermediates (triose phosphate?, glucose?) are transported across the amyloplast membranes during starch degradation.

The fact that the rate of O_2 consumption during sucrose starvation is always lower than the uncoupled rate strongly suggests that the rate at which sycamore cells respire is limited by the availability of ADP for oxidative phosphorylation (see Jacobus *et al.*, 1982). In support of this suggestion we have observed that the cytoplasmic concentration of ADP at all stages of sucrose starvation is small. In other words, the absolute concentration of ADP available for uptake into the mitochondria, which reflects the rate of metabolism, controls the rate of respiration. The decrease in the uncoupled rate of O_2 consumption observed during the course of sucrose starvation is not easily interpretable. However, such an observation could indicate that the overall capacity of the plastidic glycolytic pathway is not sufficient to sustain the maximal rate of O_2 consumption by the mitochondria. When starch breakdown replaces sucrose breakdown, the uncoupled rate of respiration is probably limited by the availability of substrate for mitochondrial respiration and not by the overall capacity of the respiratory chains. In support of this suggestion, the initial uncoupled rate of O_2 consumption by sucrose-starved cells is recovered after addition of sucrose to the medium (Fig. 4). However, further work is needed to clarify this point.

Finally, the results presented here raise the problem of sucrose movement between the vacuole and the cytoplasm. Some previously obtained data suggest the existence, on the tonoplast, of an active sucrose-transport system (for a review, see Matile & Wiemken, 1976). Although there is no clear understanding of the molecular nature of any transport process, it is possible that cytoplasmic metabolites such as sucrose and glucose 6-phosphate could regulate the movement of sucrose across the tonoplast. Likewise, the intravacuolar sucrose concentration could strongly modulate its rate of efflux and therefore the rate of cytoplasmic glycolysis.

This work was supported in part by a Research Grant from the 'Centre National de la Recherche Scientifique' (U.A. 576: Interactions Plastiques-Cytoplasme-Mitochondries).

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