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Regulation of sucrose and starch metabolism in potato tubers in response to short-term water deficit

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Abstract. To investigate the effect of water stress on carbon metabolism in growing potato tubers (*Solanum tuberosum* L.), freshly cut and washed discs were incubated in a range of mannitol concentrations corresponding to external water potential between 0 and −1.2 MPa. (i) Incorporation of [^{14}C]glucose into starch was inhibited in water-stressed discs, and labeling of sucrose was increased. High glucose overrode the changes at low water stress (up to −0.5 MPa) but not at high water stress. (ii) Although [^{14}C]sucrose uptake increased in water-stressed discs, less of the absorbed [^{14}C]sucrose was metabolised. (iii) Analysis of the sucrose content of the discs confirmed that increasing water deficit leads to a switch, from net sucrose degradation to net sucrose synthesis. (iv) In parallel incubations containing identical concentrations of sugars but differing in which sugar was labeled, degradation of [^{14}C]sucrose and labeling of sucrose from [^{14}C]glucose and fructose was found at each mannitol concentration. This shows that there is a cycle of sucrose degradation and resynthesis in these tuber discs. Increasing the extent of water stress changed the relation between sucrose breakdown and sucrose synthesis, in favour of synthesis. (v) Analysis of metabolites showed a biphasic response to increasing water deficit. Moderate water stress (0–200 mM mannitol) led to a decrease of the phosphorylated intermediates, especially 3-phosphoglycerate (3PGA). The decrease of metabolites at moderate water stress was not seen when high concentrations of glucose were supplied to the discs. More extreme water stress (300–500 mM mannitol) was accompanied by an accumulation of metabolites at low and high glucose. (vi) Moderate water stress led to an activation of sucrose phosphate synthase (SPS) in discs, and in intact tubers. The stimulation involved a change in the kinetic properties of SPS, and was blocked by protein phosphatase inhibitors. (vii) The amount of ADP-glucose (ADPGlc) decreased when discs were incubated on 100 or 200 mM mannitol. There was a strong correlation between the in vivo levels of ADPGlc and 3PGA when discs were subjected to moderate water stress, and when the sugar supply was varied. (viii) The level of ADPGlc increased and starch synthesis was further inhibited when discs were incubated in 300–500 mM mannitol. (ix) It is proposed that moderate water stress leads to an activation of SPS and stimulates sucrose synthesis. The resulting decline of 3PGA leads to a partial inhibition of ADP-glucose pyrophosphorylase and starch synthesis. More-extreme water stress leads to a further alteration of partitioning, because it inhibits the activities of one or more of the enzymes involved in the terminal reactions of starch synthesis.

Key words: Adenosine 5′-diphosphoglucose – Starch synthesis – *Solanum* (carbon metabolism, tuber) – Sucrose-phosphate synthase – Sucrose metabolism (futile cycle) – Water stress

Introduction

Storage of starch and growth occur simultaneously in growing potato tubers (Reeve et al. 1973; Schneiders et al. 1988). The unloading of sucrose and its conversion to starch therefore has to be coordinated with the synthesis of other cell components, and with the maintenance of a suitable turgor for cell growth and expansion (see Patrick 1990). The water content of the tubers can fluctuate by up to 10–20% every day (Baker

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Abbreviations: ADPGlc = ADP-glucose; AGPase = ADP-glucose pyrophosphorylase; Fru6P = fructose-6-phosphate; Glc6P = glucose-6-phosphate; 3PGA = 3-phosphoglycerate; P_i = inorganic phosphate; SPS = sucrose-phosphate synthase; UDPGlc = uridine-5′-diphosphoglucose; V_{max} = activity of SPS assayed in the presence of saturating hexose-phosphate concentration; V_{sel} = activity of SPS assayed in the presence of limiting hexose-phosphate concentration and inhibitor (P_i) included

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and Moorby 1969; Schneiders et al. 1988; Plodowska et al. 1989), depending on the rates of transpiration and the availability of water in the soil. Sucrose is unloaded symplasmically into the parenchyma cells (Oparka et al. 1992) at a local concentration of the order of 0.5 M before being converted into cell components, of which starch is the major component (Oparka and Wright 1988a,b; Geigenberger and Stitt 1993). The conversion of sucrose to starch represents a potentially important site at which tubers could regulate their internal osmotic potential, their water relations, and growth.

Soluble sugars accumulate in the leaves of many species (Munns and Weir 1981; Morgan 1984; Zrenner and Stitt 1991), including potato (Moorby et al. 1975), when they experience water stress. In leaves, water deficits lead to a stimulation of sucrose synthesis and inhibition of starch synthesis (Quick et al. 1989; Zrenner and Stitt 1991), and also stimulate starch breakdown (Stewart 1971; Zrenner and Stitt 1991). This alteration of partitioning has been attributed to activation of sucrose phosphate synthase (SPS) by reversible protein phosphorylation (Quick et al. 1989; Zrenner and Stitt 1991; Huber et al. 1992; Huber and Huber 1996).

There is evidence that turgor regulates the rate of starch synthesis in growing potato tubers (Oparka and Wright 1988a,b; Oparka et al. 1990; Hnilo and Okita 1989), but the mechanisms involved have not yet been investigated. In the following article, our first aim was to analyse the effect of water stress on the fluxes of carbon in discs from growing tubers. As in many other plant tissues (Geigenberger and Stitt 1991; MacRae et al. 1992; Hill and ap Rees 1995), there is a cycle of sucrose degradation and resynthesis in growing tubers, that allows sensitive regulation of the rate of sucrose mobilisation (Geigenberger and Stitt 1993). We have therefore investigated whether water stress affects sucrose synthesis, as well as sucrose breakdown and starch synthesis. Our second aim was to identify the enzymatic step(s) at which the fluxes are being regulated. To do this, we have investigated the activity and regulation of SPS, and the levels of metabolites in the pathway from sucrose to starch, including UDPglucose (UDPGlc), hexose phosphates, 3-phosphoglycerate (3PGA) and ADPglucose.

Materials and methods

Plant material. Potato (*Solanum tuberosum* L. cv. Desirée) plants (Saatzucht Fritz Lange, Bad Schwartau, Germany) were grown in a glasshouse in 25-l pots in soil supplemented with slow-release fertiliser pellets (Plantacote, Spiess & Sohn, Kleinkarlbach, Germany), with supplementary illumination (16 h light, 250 μmol photons $\cdot m^{-2} \cdot s^{-1}$). Growing tubers from 10- 12-week-old daily-watered plants were used for the experiments. Such tubers have high activities of sucrose synthase (above 1000 μmol $\cdot$ g $FW^{-1} \cdot h^{-1}$) which is taken as an indicator for rapidly growing tubers (Merlo et al. 1993).

Labelling experiments with discs. Labelling experiments were carried out with tuber slices cut directly from growing tubers attached to the fully photosynthesising mother plant, as in Geigenberger and Stitt (1993). Discs (diameter 8 mm, thickness 2 mm) were cut and washed 3 times in 20-fold excess medium, containing 10 mM 2-(N-morpholino)ethanesulfonic acid (Mes)-KOH (pH 6.5). After 90 min preincubation of the discs in the appropriate medium (12 discs in a volume of 5 ml) including different sugars (see legends for more details), U-[^{14}C]glucose, U-[^{14}C]fructose or U-[^{14}C]sucrose (Amersham-Buchler, Braunschweig, Germany) were added (specific activities differed depending on the experiment, see legend of Table 4) and incubation continued for another 2 h. Incubations were done in Erlenmeyer flasks (volume 100 ml) shaken at 90 rpm to maintain aerobic conditions.

Since leakage of sucrose from the discs into the medium could be a problem in this kind of experiment (Viola 1996), control experiments were done by incubating discs without external sugars and monitoring the loss of sucrose into the medium. The initial washing procedure removed ca. 19.8% of the internal sucrose from the freshly cut discs (16.7%, 2.1% and 0.9% were removed in the subsequent washing steps, respectively). During the following 3.5-h incubation, another 3–4% of the inital sucrose was lost from the discs, independent of the concentration of osmoticum in the medium (data not shown).

Fractionation of ^{14}C-labelled tissue extracts. Incubated tissue slices were washed 3 times in buffer (12 discs per 100 ml), extracted in 80% (v/v) ethanol at 80 °C (10 ml per 3 discs), re-extracted in 50% (v/v) ethanol (5 ml per 3 discs), the combined supernatants dried under vacuum at 40 °C, taken up in 1 ml H_2O (''soluble fraction''), and separated into neutral, anionic, and basic fractions by ion-exchange chromatography as in Quick et al. (1989). The insoluble material left after ethanol extraction was homogenised using mortar and pestle, taken up in 1.5 ml water and counted for starch. In discs from growing tubers, starch accounts to over 90% of the label in the insoluble fraction (Geigenberger and Stitt 1993).

To measure phosphate esters, samples (150 μl) of the soluble fraction were incubated in 50 μl buffer (10 mM Mes-KOH, pH 6.0) with or without 1 unit potato acid phosphatase (Grade II; Boehringer, Mannheim, Germany) for 3 h at 37 °C, boiled for 2 min, and analysed by ion-exchange chromatography (see above). Incubation without enzyme did not influence the proportion of radioactivity in each fraction. Label lost from the anion fraction after treatment with enzyme was assumed to have been present as phosphate esters.

The neutral fraction (3.5 ml) was freeze-dried, taken up in 75 μl water, and further separated by thin-layer chromatography (Geigenberger and Stitt 1993), developing eight times with ethyl acetate/pyridine/water (100:35:25, by vol.), and quantifying using a linear analyser (Tracemaster 20, Berthold, Bad Wildbad, Germany). Peaks were identified using radiolabelled standards.

Metabolite analysis. Tissue slices were frozen in liquid N_2, taking care that the slices fell into liquid N_2 immediately after being cut from an intact tuber, or being sampled after incubation. After extraction of frozen material (0.5 g FW) with trichloroacetic acid, pyrophosphate (PPi), hexose-phosphates; 3PGA and UDPGlc were measured as in Jelitto et al. (1992). The recovery of small, representative amounts of each metabolite through the extraction, storage, and assay procedures has been documented (Hajirezaei and Stitt 1991; Jelitto et al. 1992). Soluble sugars were measured according to Geigenberger and Stitt (1993).

The ADPGlc, ATP and ADP in trichloroacetic acid extracts were measured by high-pressure liquid chromatography (HPLC), using a Kontron HPLC system (Kontron, Eching, Germany) fitted with a Partisil-SAX anion exchange column (according to Geigenberger et al. 1994). After injecting 20 μl of extract (equivalent to 90 mg FW^{-1}), the column was washed for 12 min with 92.5% buffer A (10 mM $NH_4H_2PO_4$, adjusted to pH 2.8 with HCl): 7.5% buffer B (750 mM $NH_4H_2PO_4$, adjusted to pH 3.7), then for 3 min with a linear gradient from 7.5% B to 90% B, then for 10 min with a linear gradient from 90% B to 100% B, and then for 7 min with 100% B. The column was equilibrated for 1 min with a linear gradient from 100% B to 7.5% B and then for 10 min with 7.5% B. The flow rate was 1 ml $\cdot$ min^{-1}. The retention time of ADPGlc was 9 min, compared to 14 min for UDPGlc. The buffers were made with

freshly bidistilled water and 'suprapure'-grade $NH_4H_2PO_4$ (low absorbance at 254 nm; Merck, Darmstadt, Germany). Eluted compounds were detected by A_{254} and identified and quantified by comparison with added, authentic nucleotide standards. Recovery of a small representative amount of ADPGlc during extraction, storage and analysis is documented in Geigenberger et al. (1994).

Analysis of enzyme activities. Activity of SPS was measured by an isotopic assay as in Geigenberger and Stitt (1993) or by the anthrone method according to Huber et al. (1989). Samples for assay of SPS were frozen in liquid nitrogen, homogenised in buffer (0.28 g FW · ml^{-1}): 50 mM 3-(N-morpholino)propane-sulfonic acid (Mops-KOH, pH 7.4), 12 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM dithiothreitol (DTT), 1 ml · l^{-1} Triton X-100, 20 g · l^{-1} polyvinylpolypyrrolidone, 1 mM benzamidine and 1 mM ε-aminocaproic acid, centrifuged (8000 *g*, 5 min), 450 µl of the supernatant spin-desalted on a mini-column (filled with 5 ml Sephadex G-25, Pharmacia, pre-equilibrated with 50 mM Mops-KOH (pH 7.4), 12 mM $MgCl_2$, 1 mM DTT), and immediately assayed as in Huber et al. (1989), adding 45 µl sample to 25 µl buffer [final concentrations: 50 mM Mops-KOH (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA] containing (V_{max} assay) 12 mM fructose-6-phosphate (Fru6P), 36 mM glucose-6-phosphate (Glc6P) and 6 mM UDPGlc, or (V_{sel} assay) 2 mM Fru6P, 6 mM Glc6P, 6 mM UDPGlc and 5 mM phosphate. The reaction was stopped after 10 min at 25 °C with 70 µl 300 g · l^{-1} KOH, and the mixture heated (10 min, 95 °C). After addition of 1 ml 1.4 g · l^{-1} anthrone in 83–85% H_2SO_4, the mixture was incubated for 20 min at 40 °C and its absorbance measured at 620 nm. Control incubations were carried out from which Glc6P and Fru6P were omitted.

Results

Water stress stimulates sucrose synthesis and inhibits starch synthesis. Freshly cut discs from growing potato tubers were incubated in 10 mM Mes-KOH (pH 6.5) buffer, or on buffer plus 100, 200, 300, and 500 mM mannitol, corresponding to an external water potential of 0, −0.24, −0.48, −0.72, and −1.2 MPa, respectively. This provides a simplified experimental system, in which the water potential can be reproducibly modified, and uniform material obtained for comparison of fluxes, metabolites and enzyme activities. Discs incubated without mannitol gained slightly in fresh weight (7–10%, see Fig. 1A, Fig. 2A, and legends to Fig. 3) compared to their weight immediately after cutting them from the tuber, implying that turgor had increased. Incubation on 100, 200, 300 and 500 mM external osmoticum led to a decrease of fresh weight of 1–4%, 4–8%, 12–22% and 22–33% (Fig. 1A, Fig. 2A, legends to Fig. 3, and data not shown). Plasmolysis occurred between 300 and 500 mM mannitol (data not shown, see also Oparka and Wright 1988b). The changes between 0 and 200 mM mannitol are representative of the fluctuations experienced by tubers in the field (see Introduction). The 300 and 500 mM treatments represent a more-extreme stress. Because the fresh weight was changed by the treatment, most of the following results are expressed on a disc basis. Similar changes of fresh weight were found, irrespective of whether 5 or 50 mM glucose was included. Since glucose is absorbed, it will not act as a true osmoticum unless very high concentrations are added.

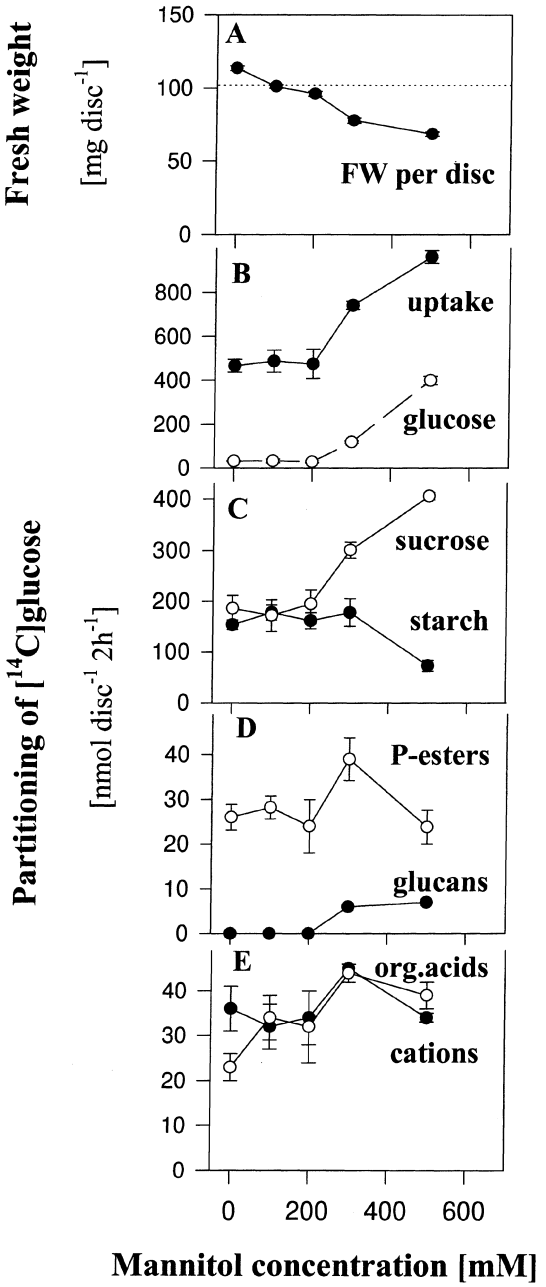


Fig. 1A–E. Metabolism of 50 mM [^{14}C]glucose by potato tuber discs from growing tubers, incubated at different external osmotica. Growing tubers were harvested from 10-week-old plants, and discs (2 mm thick, 8 mm diameter, ca. 0.1 g FW) were cut, washed 3 times in buffer, preincubated for 1.5 h in 5 ml medium containing mannitol osmoticum as in the x-axis plus 50 mM glucose. Following addition of [^{14}C]glucose (final specific activity 0.87 Bq · $nmol^{-1}$), the discs were then incubated for a further 2 h, washed 3 times, extracted, and analysed. **A** Change of fresh weight; FW of discs immediately after cutting is indicated by a *dotted line*. **B** Sucrose uptake (●) and label remaining in glucose (○). **C** Label incorporation into sucrose (○) and starch (●). **D** Label incorporation into phosphorylated intermediates (○) and glucans (●). **E** Label incorporation into other anionic compounds (○) and cations (●). The results show the rate of use of the externally supplied sugar (see below for calculation of the absolute fluxes), and are given as the mean ± SE (n = 4)

After thorough washing to remove broken cells, and a 1.5-h preincubation, the discs were supplied with either 50 mM (Fig. 1) or 5 mM (Fig. 2) [^{14}C]glucose for a further 2 h. These treatments were used to compare the effect of water stress on starch and sucrose synthesis under conditions of low and high carbohydrate supply, respectively. Glucose was taken up four to five times more quickly with 50 mM than with 5 mM glucose (compare Figs. 1B and 2B). Glucose uptake was not stimulated at moderate water stress, but did increase slightly at 300 and 500 mM mannitol. Partitioning to starch and sucrose was altered by water stress (Figs. 1C, 2C). The sensitivity depended on the amount of glucose supplied. When 50 mM glucose was supplied, a relatively large proportion of the absorbed label was already converted to sucrose in the discs incubated without external osmoticum. Moderate water stress (between 0 and 200 mM mannitol) did not have a large effect on partitioning. Higher concentrations of mannitol inhibited labeling of starch and stimulated labeling of sucrose (Fig. 1C). When 5 mM glucose was supplied, less label was incorporated into sucrose in the discs incubated without osmoticum. There was a marked shift of partitioning towards sucrose even at 100 and 200 mM mannitol. This shift continued at higher mannitol concentrations (Fig. 2C). At both glucose concentrations, labeling of phosphate esters did not change significantly in response to water stress (Figs. 1D, 2D). Labeling of organic acids and cations increased up to 300 mM mannitol, and declined slightly at 500 mM mannitol (Figs. 1E, 2E). Glucans were not significantly labeled at low mannitol, but were labeled at 300 and 500 mM mannitol (Figs. 1D, 2D).

Water stress reduces sucrose degradation. In an analogous experiment, 20 mM sucrose was supplied to investigate the impact of water stress on sucrose breakdown. To allow simultaneous measurement of sucrose synthesis in identical conditions, 10 mM glucose and 10 mM fructose were provided simultaneously in the medium. Three parallel incubations were carried out, in which tracer amounts of [^{14}C]sucrose (Fig. 3A–C), [^{14}C]glucose (Fig. 3D–F), or [^{14}C]fructose (Fig. 3G–I) were included.

Uptake of [^{14}C]sucrose doubled between 0 and 300 mM mannitol (Fig. 3A), as previously seen for potato tuber discs (Oparka and Wright 1988a,b) and other tissues (Patrick 1990). However, less of the absorbed [^{14}C]sucrose was metabolised. The proportion of the absorbed label that remained as sucrose rose from 61% in water-incubated discs, to 67%, 73%, 84%, and 85% in discs incubated on 100, 200, 300 and 500 mM mannitol (calculated from the data in Fig. 3A).The total amount of label metabolised also decreased (Fig. 3B). This inhibition of sucrose mobilisation was accompanied by a twofold decrease of label incorporation into starch (Fig. 3B). Label incorporation into the other cell fractions was not markedly affected (Fig. 3C).

In the parallel incubations with labeled glucose (Fig. 3D–F) and fructose (Figs. 3G–I) hexose uptake was not increased markedly by moderate water stress (Fig. 3D, G), and water stress led to increased labeling of sucrose, and decreased labeling of starch from both reducing sugars (Fig. 3E,H) as already seen in Figs.1 and 2. At all

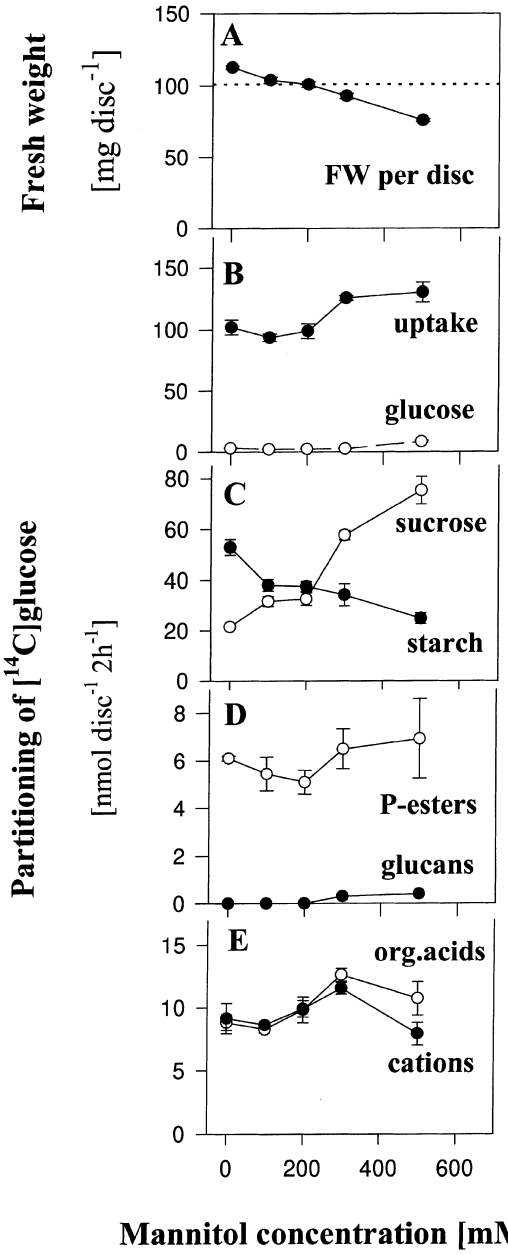


Fig. 2A–E. Metabolism of 5 mM [^{14}C]glucose by potato tuber discs from growing tubers, incubated at different external osmotica. Growing tubers were harvested from 12-week-old plants, and discs (2 mm thick, 8 mm diameter) were cut, washed 3 times in buffer, preincubated for 1.5 h in medium containing mannitol osmoticum as in the x-axis plus 5 mM glucose. Following addition of [^{14}C]glucose (final specific activity 5.05 Bq · nmol^{-1}), the discs were then incubated for a further 2 h, washed 3 times, extracted, and analysed. **A** Change of fresh weight; FW of discs immediately after cutting is indicated by a *dotted line*. **B** Sucrose uptake (●) and label remaining in glucose (○). **C** Label incorporation into sucrose (○) and starch (●). **D** Label incorporation into phosphorylated intermediates (○) and glucans (●). **E** Label incorporation into other anionic compounds (○) and cations (●). The results show the rate of use of the externally supplied sugar (see below for calculation of the absolute fluxes) and are given as the mean ± SE (n = 4)

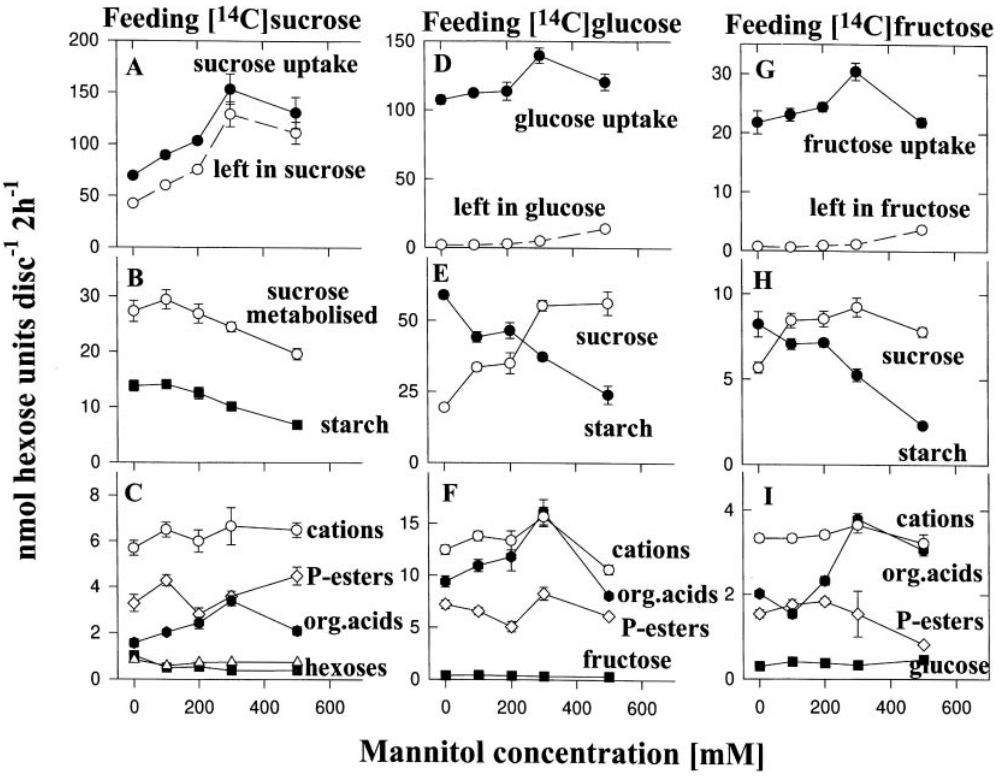


Fig. 3A–I. Metabolism of sucrose, glucose and fructose by potato tuber discs from growing tubers, incubated at different external osmotica. Growing tubers were harvested from 12-week-old plants, and discs (2 mm thick, 8 mm diameter) were cut, washed 3 times in buffer, preincubated for 1.5 h in medium containing mannitol osmoticum as in the x-axis plus 20 mM sucrose, 10 mM glucose, and 10 mM fructose. Three parallel incubations then received either [^{14}C]sucrose (final specific activity 1.85 Bq · nmol^{-1}, **A–C**), or [^{14}C]glucose (final specific activity 7.5 Bq · nmol^{-1}, **D–F**), or [^{14}C]fructose (final specific activity 7.5 Bq · nmol^{-1}, **G–I**). The discs were then incubated for a further 2 h, washed 3 times, extracted, and analysed. **A** Sucrose uptake (●) and label remaining in sucrose (○). **B**. Label mobilised out of sucrose (○, calculated from **A**) and incorporation into starch (●). **C** Label incorporation into phosphorylated intermediates (◇), other anionic compounds (●), cations (○), glucose (△) and fructose (■). **D** Glucose uptake (●) and label remaining in glucose (○). **E** Label incorporation into sucrose (○) and starch (●). **F** Label incorporation into phosphorylated intermediates (◇), other anionic compounds (●), cations (○) and fructose (■). **G** Fructose uptake (●) and label remaining in fructose (○). **H**. Label incorporation into sucrose (○) and starch (●). **I** Label incorporation into phosphorylated intermediates (◇), other anionic compounds (●), cations (○) and glucose (■). The results show the rate of use of the externally supplied sugar (see below for calculation of the absolute fluxes). All results are expressed on a disc basis (fresh weights were 100 mg for freshly cut discs, and 106, 96, 93, 79 and 69 mg for discs incubated in 0, 100, 200, 300 and 500 mM mannitol, respectively), and are given as the mean ± SE (n = 4), except where the error bars are smaller than the symbol

of the mannitol concentrations used, there was simultaneous degradation of [^{14}C]sucrose (Fig. 3B), and labeling of sucrose from [^{14}C]glucose and fructose (Fig. 3E, H). This shows that there is a cycle of sucrose synthesis and degradation in these tuber discs. As the extent of the water stress is increased, the relation between sucrose breakdown and sucrose synthesis changes, in favour of synthesis.

Water stress leads to a switch from net sucrose degradation to net sucrose synthesis. The net rate of sucrose mobilisation will depend on the balance between the rate of synthesis and the rate of degradation. The end result can be determined in discs by subtracting the content of sucrose at the beginning from that at the end of the incubation (Table 1).

The net rate of sucrose mobilisation will be overestimated if sucrose leaks from the discs into the medium during incubation. To check this, we did control experiments, where discs were incubated without supplying sugars in the medium. Analysis of the medium revealed that 3.7–4.8% of the initial amount of sucrose in the discs is lost into the medium during the 3.5-h incubation, and that this is not dependent on the mannitol concentration provided (data not shown). We added this amount of sucrose to the sucrose values measured in the discs at the end of the incubation to correct the data for sucrose leakage from the discs. The results in Table 1 will underestimate the net rate of sucrose mobilisation in incubations containing sucrose in the medium (Fig. 3), because some sucrose will be taken up into the discs. The amount of sucrose taken up (see Fig. 3A) must be added to the difference between the amount of sucrose in discs at the beginning and at the end of the incubation (see Table 1) to give the actual rate of sucrose mobilisation (see later for the corrected values).

When discs were incubated in water or low mannitol concentrations there was a net depletion of sucrose, at a rate of about 3.5 μmol hexose · g FW^{-1}h^{-1} (Table 1, data calculated on a fresh weight basis). This reflects the

Table 1. Water stress decreases the net sucrose depletion in discs. Discs were cut from growing tubers and incubated for 3.5 h with different carbohydrate supply. When discs are incubated without sugar or with external glucose, the change of their sucrose content at the beginning and the end of the incubation period (3.5 h) is a direct estimate of the net rate of sucrose degradation. Since 4.5% of the inital amount of sucrose leaks from the discs during incubation (data not shown), this amount was added to the sucrose values obtained in discs harvested at the end of incubation. In the experiment where sucrose was included, the sucrose content is also affected by sucrose uptake from the medium. The changes in sucrose content obtained in this experiment, therefore, underestimate the net rate of sucrose degradation in these discs (corrected values see Table 5). Data are the mean ± SE of 4–5 different incubations

Carbohydrate supply	Decrease in sucrose content (nmol hexose equiv $disc^{-1}$ 3.5 h^{-1}) of discs incubated for 3.5 h in a solution containing mannitol at				
	0	100 mM	200 mM	300 mM	500 mM
No sugars	1042 ± 190	712 ± 83	1019 ± 132	79 ± 7	−64 ± 6
0.5 mM Glc	1356 ± 311	1783 ± 136	88 ± 19	−149 ± 28	−571 ± 84
5 mM Glc	610 ± 253	923 ± 202	433 ± 113	208 ± 63	−946 ± 274
50 mM Glc	53 ± 24	194 ± 38	−289 ± 46	−5 ± 32	−601 ± 70
20 mM Suc + Glc + Fru	600 ± 77	830 ± 103	560 ± 62	400 ± 43	40 ± 6

net metabolism of sucrose to starch, and other cell components. The net rate of mobilisation was decreased to about 0.35 $\mu mol \cdot g\ FW^{-1}h^{-1}$ when high levels of glucose were provided in the medium. Net sucrose mobilisation was also inhibited when mannitol was included in the medium, confirming that water stress modifies sucrose metabolism in these tuber discs. There was no inhibition between 0 and 100 mM mannitol, but a marked inhibition was found when the mannitol concentration was increased to 200, 300 and 500 mM. With 500 mM mannitol, net mobilisation was almost totally inhibited (in the presence of 20 mM sucrose plus hexoses) or even reversed to net sucrose synthesis (in the presence of 5 or 50 mM glucose).

Sucrose synthesis in water-stressed discs occurs via sucrose-phosphate synthase. Label can enter sucrose via SPS, or via the readily reversible reaction catalysed by sucrose synthase. Over half of the label incorporation can occur via sucrose synthase in non-stressed discs and intact tubers (Geigenberger and Stitt 1993). Label incorporation via sucrose synthase can be distinguished, because it leads to preferential incorporation of label from fructose, compared to glucose (incoming label from fructose is directly incorporated into the fructosyl moiety of sucrose by sucrose synthase, without being diluted by other unlabelled exogenous or endogenous sources of carbon that are entering the hexose-phosphate pool; see Geigenberger and Stitt 1993)

Comparison of Fig. 3E and 3H reveals that labeling of sucrose from glucose and from fructose is affected differentially by water stress (summarised in Table 2). When discs were incubated without mannitol a larger proportion of the absorbed [^{14}C]fructose (26%) than the absorbed [^{14}C]glucose (17%) was converted to sucrose, as previously observed (Geigenberger and Stitt 1993). When mannitol was included in the medium, labeling of sucrose from [^{14}C]fructose was stimulated less than labeling from [^{14}C]glucose. As a result, sucrose was not preferentially labeled from fructose at high mannitol concentrations. This indicates that sucrose is being synthesised mainly via SPS in the water-stressed discs.

Table 2. Water stress has a differential effect on the metabolism of [^{14}C]glucose and [^{14}C] fructose to sucrose. The results are calculated from the data in Fig. 3

Isotope supplied	^{14}C in sucrose (as a % of absorbed label) in discs incubated in a solution containing mannitol at				
	0	100 mM	200 mM	300 mM	500 mM
[^{14}C]Glucose	17	30	31	40	47
[^{14}C]Fructose	26	37	35	37	36

Changes of phosphorylated intermediates. To identify potential sites for regulation, metabolites were measured in parallel samples from the experiments of Figs. 1–3. Non-stressed discs supplied with 20 mM sucrose plus hexoses (Fig. 4G–I, corresponding to the labeling data in Fig. 3) contained slightly lower levels of metabolites than those supplied with 5 mM glucose alone (Fig. 4D–F, corresponding to the labeling data in Fig. 2). Replicate experiments confirmed that hexose phosphates and 3PGA decrease when discs are supplied with up to 200 mM sucrose (data not shown).

The response of metabolites to water stress in the experiments of Fig. 2 (with 5 mM glucose) and Fig. 3 (with 20 mM sucrose, 10 mM glucose, fructose) can be separated into two phases. (i) As the mannitol concentration was increased from 0 to 100 and 200 mM, there was a small decrease of Glc6P (Fig. 4D,G), Fru6P (Fig. 4E,H) and glycerate-3-phosphate (3PGA) (Fig. 4F,I). The changes of Glc6P and 3PGA were significant in both experiments ($P < 0.05$). (ii) As the mannitol concentration was further increased to 300 or 500 mM, all these metabolites increased again. This increase was significant in both experiments. The UDPGlc decreased slightly but significantly ($P < 0.05$) between 0 and 200 mM mannitol, and then increased again at higher mannitol concentrations (Fig. 5A; similar results were also obtained for 5 mM glucose, data not shown). Pyrophosphate (PPi) decreased above 100 mM mannitol (Fig. 5C). There was a gradual decrease of ATP across the entire range of mannitol concentrations in the presence of 20 mM sucrose,

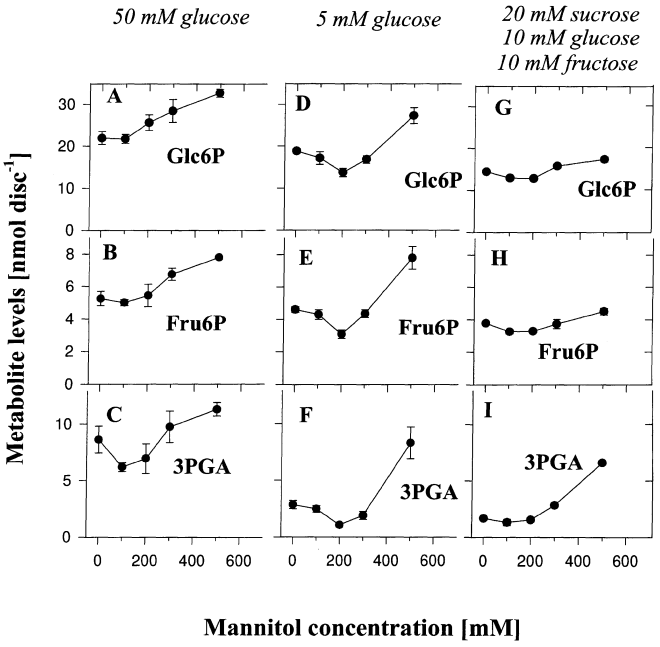


Fig. 4A–I. Levels of phosphorylated intermediates. In parallel samples from the labelling experiments of Figs 1–3., supplied with 50 mM glucose (**A–C**), 5 mM glucose (**D–F**), or 20 mM sucrose, 10 mM glucose and 10 mM fructose (**G–I**), extracts were prepared and assayed for Glc6P (**A, D, G**), Fru6P (**B, D, H**), and glycerate-3-phosphate (**C, F, I**). All results are expressed on a disc basis (for fresh weights see Figs. 1–3), and are given as the mean ± SE (n = 4), except where the error bars are smaller than the symbol

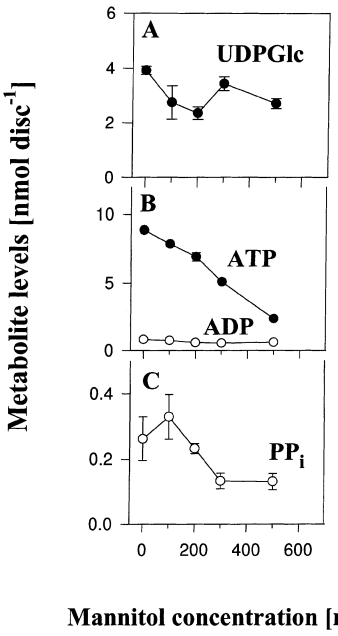


Fig. 5A–C. Levels of UDP-Glc (**A**), ATP (●), ADP (○) (**B**) and inorganic pyrophosphate (**C**). Extracts were prepared and assayed in parallel samples from the labelling experiment of Fig. 3, supplied with 20 mM sucrose, 10 mM glucose and 10 mM fructose. The results are expressed on a disc basis and are given as the mean ± SE (n = 4), except where the error bars are smaller than the symbol

10 mM glucose and fructose (Fig. 5B), and at 300–500 mM mannitol concentrations in the presence of 5 mM glucose (data not shown).

The high rates of sugar uptake in the discs supplied with 50 mM glucose (Figs. 4A–C, corresponding to the labeling data in Fig. 1) resulted in a general increase of phosphorylated intermediates, especially 3PGA (compare Fig. 4A–C with Fig. 4D–F and Fig. 4G–I). Water stress led to smaller changes of metabolite levels than in the presence of 5 mM glucose (Fig. 4A–C). When the mannitol concentration was increased from 0 to 200 mM, there was no significant change of hexose-phosphates, and a relatively small decrease of 3PGA. Hexose phosphates and 3PGA increased at higher mannitol concentrations, but the increase was (in relative terms) smaller than in the other incubations. Levels of ATP increased slightly at 0–200 mM mannitol, and decreased at 300–500 mM mannitol (data not shown).

Moderate water stress led to a stimulation of sucrose synthesis (Figs. 1–3), even though the levels of the precursors remained unaltered or even decline slightly (Fig. 4). Taken together, these results indicate that SPS is stimulated by moderate water stress. The partial inhibition of starch synthesis in moderate water stress could be a consequence of the lower levels of phosphorylated intermediates, especially 3PGA, which is an important activator of ADP-glucose pyrophosphorylase (AGPase) in plants (Preiss 1988). In agreement, starch synthesis was not inhibited by moderate water stress when high concentrations of glucose were supplied, and large pools of intermediates were maintained (Fig. 1, Fig. 4A–C). The general accumulation of metabolites at higher mannitol concentrations indicates that more-severe water stress directly inhibits one or more of the reactions in the pathway of starch synthesis. These explanations were tested by investigating the effect of water stress on SPS activity, and ADPGlc levels.

Regulation of SPS. Regulation of spinach leaf SPS by reversible protein phosphorylation can be detected by assaying the enzyme in conditions in which the phosphorylated and dephosphorylated forms of the enzyme show different activities, and by using protein phosphatase inhibitors (Siegl et al. 1990; Huber et al. 1992). Incubation of discs for 3–4 h on zero or 300 mM mannitol did not have any significant effect on SPS activity when it was assayed in the presence of saturating hexose phosphates (termed the V_{max} assay, with 12 mM Fru6P, 36 mM Glc6P, 6 mM UDPGlc; Table 3). There was also no change in the amount of SPS, or the molecular weight of SPS protein, as revealed by Western blots (data not shown). However, when SPS was assayed in the presence of limiting substrates (termed the V_{sel} assay, with 2 mM Fru6P, 6 mM Glc6P, 6 mM UDPGlc and 5 mM phosphate [P_i]), incubation on 300 mM mannitol led to a 50–80% increase of activity. This increase was prevented by low concentrations of okadaic acid and calyculin A (inhibitors of protein phosphatases 1 and 2A;Table 3). The change of fresh weight occurred within 1 h (see Fig. 6A). The increase of SPS activity in the V_{sel} assay was slower, requiring 2–4 h (see Fig. 6B).

Table 3. Okadaic acid and calyculin A prevent the change of the kinetic properties of SPS activity that normally occurs when discs from growing tubers are incubated on 300 mM mannitol. In experiment 1, discs (9 mm diameter, ca. 0.13 g FW) were incubated for 3 h in medium containing, no mannitol, or 300 mM mannitol, or 300 mM mannitol plus ocadaic acid equivalent to 19 nmol · g FW^{-1} tissue. In experiment 2, discs (8 mm diameter, ca. 0.9 g FW) were incubated for 4 h in 20 mM mannitol, or 300 mM mannitol or 300 mM mannitol plus ocadaic acid (equivalent to 11 nmol · g FW^{-1} tissue), or 300 mM mannitol plus calyculin A (equivalent to 3.6 nmol · g FW^{-1} tissue). SPS was then extracted and measured in two different assay conditions containing saturating substrates (V_{max} assay; 12 mM Fru6P, 36 mM Glc6P, 6 mM UDPGlc), or limiting substrates plus inhibitor (V_{sel} assay; 2 mM Fru6P, 6 mM Glc6P, 6 mM UDPGlc, 5 mM Pi). The results are the mean ± SE (n = 3–4).

Experiment	Mannitol (mM)	Time (h)	Okadaic acid	Caliculin A	V_{max}	V_{sel}
					(nmol $disc^{-1}$ min^{-1})	
Expt.1	0	3	–	–	41.8 ± 5.9	7.5 ± 2.2
	300	3	–	–	55.6 ± 1.9	12.4 ± 0.7
	300	3	+	–	56.8 ± 2.8	9.3 ± 0.8
Expt.2	20	4	–	–	48.8 ± 0.8	13.1 ± 0.4
	300	4	–	–	47.4 ± 4.3	19.4 ± 2.1
	300	4	+	–	44.6 ± 3.6	14.6 ± 0.6
	300	4	–	+	46.4 ± 0.4	12.3 ± 0.6

Sucrose-phosphate synthase was also assayed in samples taken in parallel to the measurements of fluxes and metabolite levels (Figs. 1–4). There was no consistent effect of water stress on SPS activity in the presence of saturating substrate and activator concentrations (Fig. 7A,D,G). In the experiments in which discs were incubated in the presence of 5 mM glucose (Fig. 7E, corresponding to the material used in the tracer experiments of Fig. 2), or 20 mM sucrose plus hexoses (Fig. 7H) corresponding to the material used in the tracer experiments of Fig 3), SPS activity in the V_{sel} assay was relatively low in water-incubated discs and increased markedly as the mannitol osmoticum was increased up to 300 mM. This coincided with the stimulation of sucrose synthesis (see Figs. 2C, 3E) and the slight decrease of metabolites in this range (see Fig. 4D–I). In the presence of 50 mM glucose (Fig. 7B, corresponding to the material used in the tracer experiment of Fig. 1), SPS activity in the V_{sel} assay was already high in water-incubated discs, and did not rise further in response to water stress. This corresponds with the high initial rates of sucrose synthesis, and the absence of any further stimulation by moderate water stress in the presence of 50 mM glucose (see Fig. 1C). The ratio of activity in the two assay conditions increased in response to water stress between 0 and 300 mM mannitol in the first two conditions (Fig. 7F, I), but not when discs were supplied with 50 mM glucose (Fig. 7C). An apparent discrepancy emerges between 300 and 500 mM mannitol. Sucrose synthesis was increased in all three experiments (see Figs. 1C, 2C and 3E), but SPS activity did not increase further, or even declined slightly. In this range, however, there was a marked accumulation of metabolites, including the hexose phosphates (Fig. 4A–I).

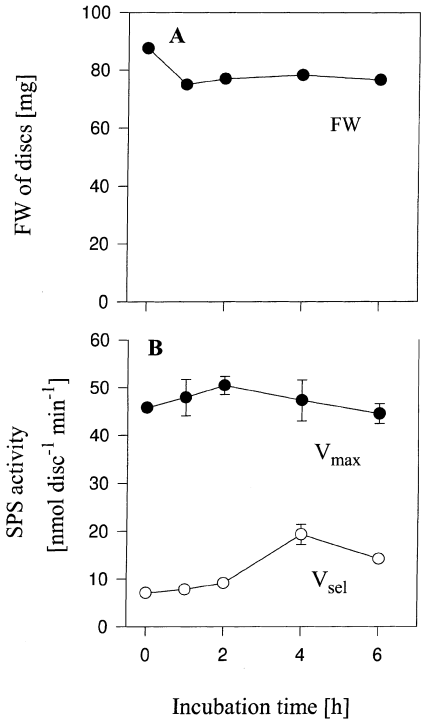


Fig. 6A–B. Time course of changes of fresh weight (**A**) and SPS activity (**B**) in tuber discs in response to 300 mM external mannitol. Tuber discs were incubated on 300 mM mannitol for 0–6 h, and extracts prepared and assayed for SPS in the presence of 12 mM Fru6P, 36 mM Glu6P and 6 mM UDPGlc (V_{max} assay, ●), or in the presence of 2 mM Fru6P, 6 mM Glc6P, 6 mM UDPGlc, and 5 mM P_i (V_{sel} assay, ○). The results are given as the mean ± SE, except where the error bars are smaller than the symbol

Levels of ADPGlc. Discs supplied with 5 mM glucose (Fig. 8B) showed a biphasic response of ADPGlc to increasing water stress. There was a decrease between 0 and 200 mM mannitol, followed by a large increase at higher mannitol concentrations. A biphasic response with a fivefold decrease between water and 200 mM mannitol and a fourfold increase between 200 and 500 mM mannitol was also found in discs supplied with 1 mM glucose (data not shown). Discs supplied with 20 mM sucrose plus hexoses (Fig. 8C) showed a weaker biphasic response (Fig. 8C). The ADPGlc level in water-incubated discs was lower and the decrease in low mannitol was smaller than in the presence of glucose. There was a large increase of ADPGlc at mannitol

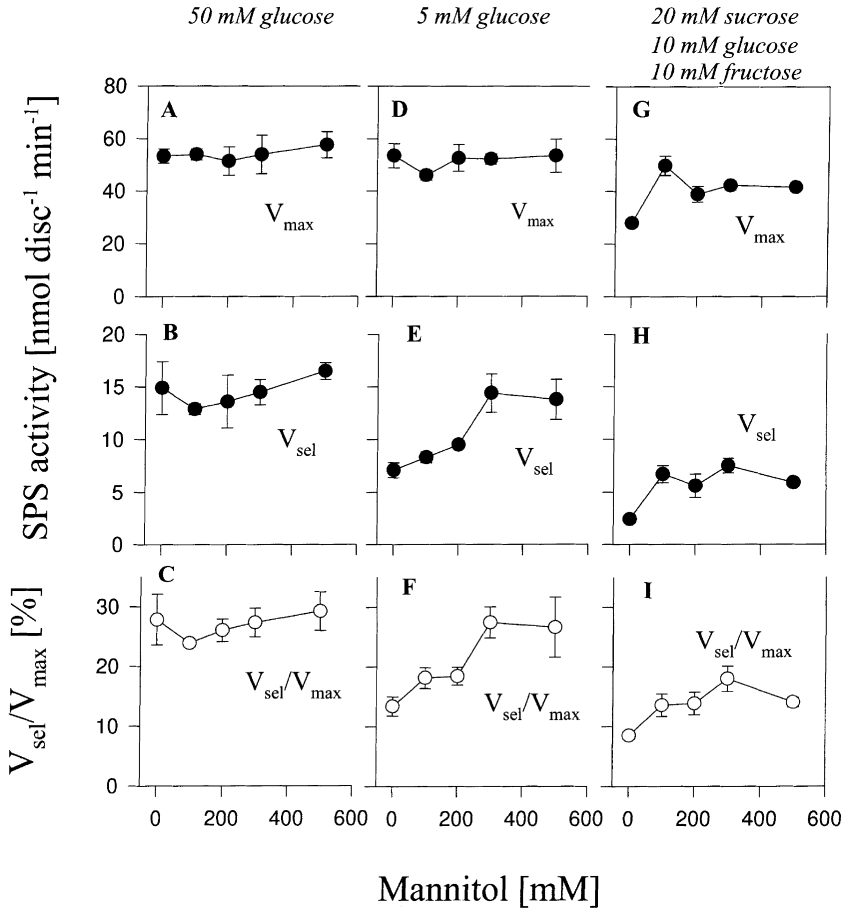
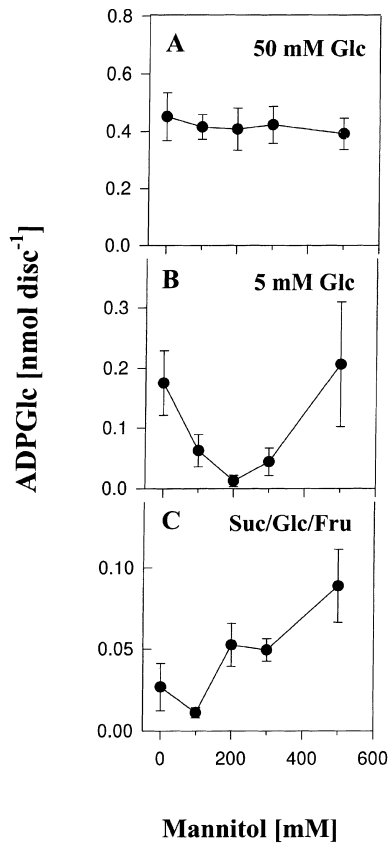


Fig. 7A–I. Effect of external mannitol concentrations and sugar supply on SPS activity. Activity was measured in samples incubated in parallel with the labeling experiments of Figs. 1–3, and harvested after 3 h incubation in the presence of various mannitol concentrations (see x-axis) and either 50 mM glucose (**A, B, C**), 5 mM glucose (**D, E, F**), or 20 mM sucrose, 10 mM glucose and 10 mM fructose (**G, H. I**). The SPS activity was assayed in V_{max} (**A, D, G**) and V_{sel} conditions (**B, E:** 2 mM Fru6P, 6 mM Glc6P, 6 mM UDPGlc, and 5 mM Pi; **H:** 1 mM Fru6P, 3 mM Glc6P, 6 mM UDPGlc, and 5 mM Pi). The results are expressed as activities, or the ratio of the activity in the two conditions (**C**, **F**, **I**), and are given as the mean ± SE (n = 3–4), except where the error bars are smaller than the symbol



concentrations above 200 mM. In discs supplied with 50 mM glucose, ADPGlc levels were very high and did not change significantly in response to increasing osmoticum (Fig. 8A).

Figure 9 compares the contents of 3PGA and ADPGlc. There is a strong correlation between the levels of 3PGA and ADPGlc in the incubations with various sugar supplies and 0 to 200 mM mannitol (Fig. 9A). This indicates that 3PGA mediates the effect of sugar supply on the ADPGlc level. It also indicates that the decrease of 3PGA is responsible for the decline of ADPGlc under moderate water stress. In the presence of higher (300 and 500 mM) mannitol concentrations, the relationship between 3PGA and ADPGlc is displaced to the right (Fig. 9B, see below for discussion).

Estimation of the rates of starch synthesis, and comparison with the ADPGlc levels. The rates of ^{14}C-incorporation into starch in Figs. 1–3 do not reveal the absolute rate of starch synthesis, because the incoming label will

Fig. 8A–C. Levels of ADPGlc. Extracts were prepared and assayed for ADPGlc in parallel samples from the labeling experiments of Figs. 1–3, supplied with 50 mM glucose **(A)**, 5 mM glucose **(B)**, and 20 mM sucrose, 10 mM glucose and 10 mM fructose **(C)**. Results are expressed on a disc basis (for FW see Figs. 1, 2 and 3), and are given as the mean ± SE (n = 4), except where the error bars are smaller than the symbol

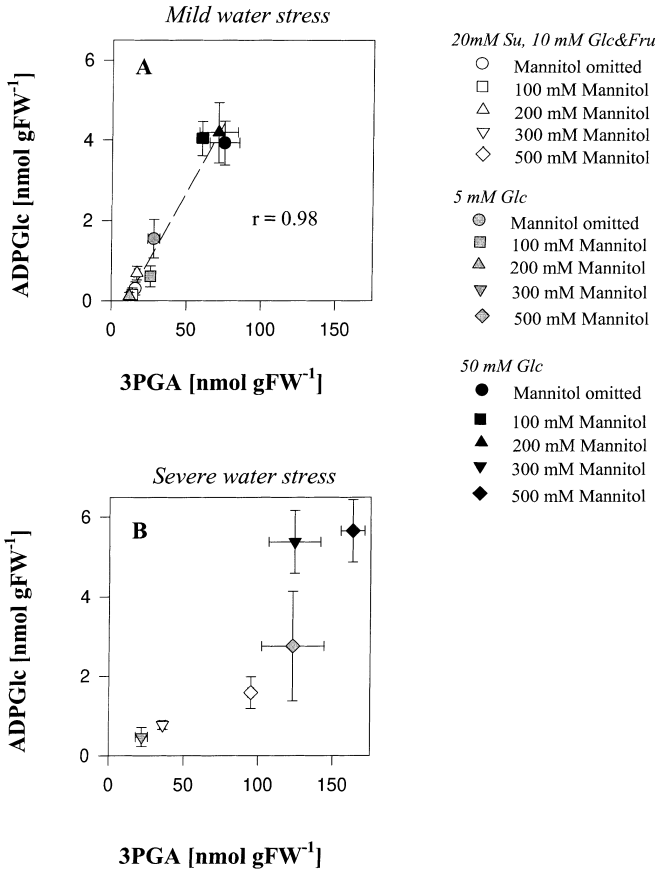


Fig. 9A–B. Relation between the level of 3PGA and ADPGlc. The results are taken from Figs. 4 and 8, and are expressed on a FW basis to adjust for possible changes in cytoplasmic volume. Results are shown for discs supplied with 50 mM glucose, with 5 mM glucose, and with 20 mM sucrose, 10 mM glucose and 10 mM fructose. **A** Data points corresponding to 0, 100, and 200 mM mannitol. (**B**) Data points corresponding to 300 and 500 mM mannitol. The results are shown as the mean ± SE, except where the error bars are smaller than the symbol

mix with unlabeled carbon deriving from internal pools in the discs. The resulting isotope dilution could lead to errors of interpretation when sets of data are compared for treatments in which different concentrations or types of sugar are provided, or the rates of mobilisation of endogenous pools are being changed. A better estimate of the absolute rate of starch synthesis can be obtained by dividing the rate of label incorporation into starch (Figs. 1C, 2C, 3B, 3E) by the specific activity of the hexose phosphates for each individual incubation.

To estimate the specific activity of the hexose phosphates, we determined the label in the pool of phosphorylated intermediates in each individual incubation, by measuring the label released by acid phosphatase treatment of the anionic fraction (Figs. 1D, 2D, 3C, 3F). This value was divided by the summed carbon in hexose phosphates and 3PGA in that incubation (Fig. 4). These intermediates represent the majority of the phosphorylated intermediates in a growing tuber (Hajirezaei et al. 1994). This approach assumes that there is isotopic equilibrium between the intermediate pools in the cytosol and plastid.

Comparison of the specific activity of the supplied sugar with that of the phosphorylated intermediates (summarised in Table 4) provides information about the rate of use of the exogenously supplied labelled sugar, compared to the rate of mobilisation of other exogenous and endogenous carbon pools. Whereas 50 mM glucose represents a major source of the carbon entering the pools of phosphorylated intermediates, 5 and 10 mM glucose only represent 22–28% and 33–41% of the total carbon entering these intermediate pools. The specific activity of the phosphorylated intermediates is also much lower than that of the supplied [^{14}C]sucrose.

The estimated rates of starch synthesis (Table 4) confirm (i) that water stress leads to a partial inhibition of starch synthesis in the experiments with 5 mM glucose and with 20 mM sucrose and hexoses, and (ii) that this inhibition is less pronounced when 50 mM glucose is supplied, and does not develop until a more extreme water stress is imposed.

The corrected values also show that similar rates of starch synthesis are reached in the presence of 5 mM glucose and 50 mM glucose. The rate of starch synthesis was about 20% lower in discs incubated with a mixture of 20 mM sucrose and hexoses, provided the rates are calculated from the labeling data with glucose. Twofold-lower rates of starch synthesis were estimated from [^{14}C]sucrose label incorporation, in an otherwise identical incubation. This anomaly may occur because the absorbed [^{14}C]sucrose enters a very large internal pool (the amount of sucrose taken up is less than 5% of the endogenous pool, data not shown). As a result, the specific activity of the internal sucrose pool will rise with time. The *average* specific activity of the endogenous sucrose pool (and the hexose phosphate pool) during the 2-h feeding experiment could be overestimated by up to twofold in our analyses, because they were made on samples taken at the end of the incubation. The rates of starch synthesis will be underestimated by a similar factor. This problem does not arise for [^{14}C]glucose, because it is metabolised without mixing with a large internal pool.

In Fig. 10, the estimated rates of starch synthesis are plotted against the ADPGlc levels for the ranges of 0 to 200 mM mannitol (Fig. 10A), and 300 to 500 mM mannitol (Fig. 10B). In Fig. 10A, half-maximal rates of starch synthesis are achieved at less than 0.6 nmol/g fresh weight, and increasing the ADPGlc content above 1.8 nmol · g FW^{-1} does not lead to any further stimulation of starch synthesis. Under more-extreme water stress (Fig. 10B), the relation between ADPGlc content and starch synthesis is displaced, and higher levels of ADPGlc are needed to attain a given rate of starch synthesis.

Estimation of the rates of sucrose cycling. Table 5 summarises the estimated rates of unidirectional sucrose synthesis and degradation, and the net rates of sucrose mobilisation. The unidirectional rates of sucrose synthesis were calculated by dividing the incorporation of label from [^{14}C]glucose into sucrose (Figs. 1C, 2C, 3E) by the specific activity of the hexose-phosphate pool (see

Table 4. Absolute rates of starch synthesis in potato discs incubated with different concentrations of mannitol, and different supplies of sugar. The fluxes are calculated from the label incorporation data (Figs. 1–3) using the specific activity of the hexose-phosphate (HP) pool in the discs to correct for isotope dilution. The specific activity of the hexose-phosphate pool was estimated by dividing the amount of label released from the anionic fraction by the action of acid phosphatase by the amount of phosphorylated intermediates (sum of hexose phosphates and 3PGA) measured in the discs (see Fig. 4). Data are normalised per disc (8 mm diameter) and a 2-h incubation period. The SE included are a maximum estimate calculated as the sum of the relative SE of each term

Experiment	Mannitol supplied mM	Specific activity of		Correction factor	Unidirectional rate of starch synthesis nmol HP disc^{-1} 2h^{-1}
		External sugar Bq · nmol^{-1}	HP-pool Bq · nmol^{-1}		
Fig. 1	0	0.867	0.617 ± 0.086	1.41	219 ± 61
(50 mM Glc)	100	0.867	0.712 ± 0.097	1.22	217 ± 37
[^{14}C] Glucose	200	0.867	0.523 ± 0.166	1.66	269 ± 123
	300	0.867	0.725 ± 0.157	1.20	213 ± 78
	500	0.867	0.387 ± 0.054	2.24	161 ± 46
Fig. 2	0	5.05	1.150 ± 0.063	4.39	232 ± 26
(5 mM Glc)	100	5.05	1.117 ± 0.237	4.52	171 ± 46
[^{14}C]Glucose	200	5.05	1.388 ± 0.208	3.64	138 ± 28
	300	5.05	1.365 ± 0.313	3.70	124 ± 38
	500	5.05	0.792 ± 0.112	6.38	152 ± 34
Fig. 3	0	7.50	2.600 ± 0.120	2.89	164 ± 11
(Glc/Fru/Suc)	100	7.50	2.667 ± 0.200	2.80	119 ± 13
[^{14}C]Glucose	200	7.50	2.083 ± 0.123	3.60	162 ± 19
	300	7.50	2.667 ± 0.078	2.92	101 ± 6
	500	7.50	1.583 ± 0.200	4.74	109 ± 28
[^{14}C]Sucrose	0	1.85	0.292 ± 0.020	6.24	97 ± 13
	100	1.85	0.430 ± 0.058	4.32	67 ± 13
	200	1.85	0.282 ± 0.040	6.56	90 ± 18
	300	1.85	0.283 ± 0.055	6.56	73 ± 16
	500	1.85	0.282 ± 0.015	6.56	49 ± 5

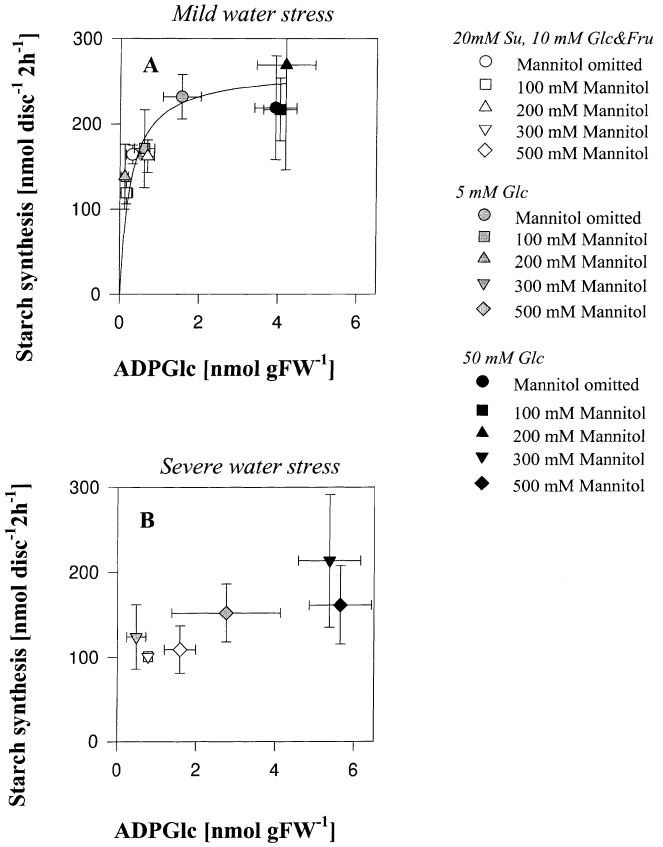


Fig. 10A–B. Relation between the ADPGlc level and the rate of starch synthesis. The results are taken from Fig 8 and Table 4. Results are shown for discs supplied with 50 mM glucose, with 5 mM glucose and with 20 mM sucrose, 10 mM glucose and 10 mM fructose. **A** Data points corresponding to 0, 100 , and 200 mM mannitol. **B** Data points corresponding to 300 and 500 mM mannitol. The results are shown as the mean ± SE, except where the error bars are smaller than the symbol

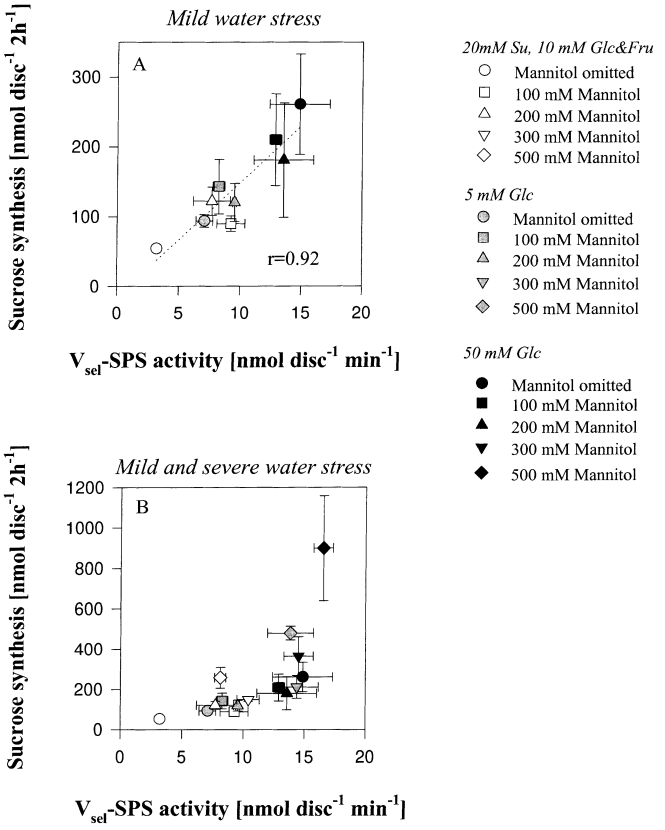
Table 4, and the last section for possible sources of error). For the experiments in which only glucose was supplied (Figs. 1, 2), the net rate of sucrose mobilisation was directly measured as the change in the sucrose content of the discs (Table 1). For the experiment in which sucrose was included (Fig. 3) the net rate of sucrose degradation was estimated as the difference between the amount of sucrose at the beginning and end of the incubation (see Table 1), plus the amount of sucrose taken up during this time interval (estimated from the data in Fig. 3A). The unidirectional rate of sucrose breakdown was estimated in all three cases as the difference between the net rate of sucrose mobilisation and the unidirectional rate of sucrose synthesis. It could not be reliably calculated from the mobilisation of [^{14}C]sucrose, because the incoming sucrose does not mix with the entire pool of sucrose in the discs (data not shown). It was therefore impossible to reliably estimate the specific activity of the metabolised sucrose pool.

The estimated fluxes around the sucrose cycle are of a similar order in the presence of 5 mM glucose, and 20 mM sucrose plus 10 mM glucose and fructose, and are about threefold higher in the presence of 50 mM glucose (Table 5). This corresponds to the higher activation of SPS in the latter conditions (see Fig. 7). The fluxes around this cycle are comparable with, or higher than, the rate of starch synthesis (see Table 4). Between 0 and 100 mM mannitol there was a small stimulation of unidirectional sucrose degradation and net sucrose degradation. The strong inhibition of net sucrose mobilisation at higher mannitol concentrations (see above) is the result of a stimulation of sucrose synthesis and an inhibition of sucrose degradation.

Figure 11 shows the relation between the rate of unidirectional sucrose synthesis and the in vitro activity of SPS, assayed in the presence of limiting substrates and P_i.

Table 5. Absolute rates of unidirectional sucrose synthesis, unidirectional sucrose degradation, and net sucrose mobilisation in discs incubated with different mannitol concentrations, and different supplies of sugar. The unidirectional rates of sucrose synthesis were estimated from the ^{14}C-incorporation from [^{14}C] glucose in sucrose (Figs. 1C, 2C, 3E), divided by the specific activity of the hexose phosphates (Table 4). The SE values were calculated by adding the relative SE, respectively. The net rate of sucrose mobilisation was directly measured as depletion of sucrose for incubations with 5 and 50 mM glucose (Table 1). For the experiment in which sucrose was included in the medium (Fig. 3) the net rate of sucrose mobilisation could not be directly measured, because the sucrose content is also affected by sucrose uptake from the medium. The net rate of sucrose degradation was estimated as the difference between the amount of sucrose at the beginning and end of the incubation (see Table 1), plus the amount of sucrose taken up during this time interval (estimated from the data in Fig. 3A). The unidirectional rate of sucrose breakdown was estimated as the sum of the rates of net sucrose mobilisation and sucrose synthesis. The data are normalised to a 2-h incubation period

Sugar supplied (mM)	Mannitol supplied (mM)	Estimated fluxes (nmol hexose · disc^{-1} · 2 h^{-1})		
		Unidir. sucrose synthesis	Net sucrose degradation	Unidir. sucrose degradation
50 mM Glc	0	261 ± 72	31 ± 14	292
	100	210 ± 66	111 ± 20	321
	200	181 ± 82	−165 ± 26	16
	300	364 ± 260	−2 ± 13	362
	500	898 ± 260	−344 ± 40	554
5 mM Glc	0	94 ± 9	349 ± 145	443
	100	143 ± 39	527 ± 115	670
	200	120 ± 27	247 ± 65	367
	300	212 ± 56	119 ± 36	331
	500	480 ± 102	−541 ± 157	−61
20 mM Suc +10 mM Glc & Fru	0	54 ± 3	412 ± 66	466
	100	90 ± 11	563 ± 78	653
	200	122 ± 20	423 ± 52	545
	300	150 ± 8	382 ± 78	532
	500	258 ± 51	153 ± 40	411



Provided the data points for 300 and 500 mM mannitol are excluded, there is a strong correlation (Fig. 11A), indicating that these changes in the kinetic properties of SPS play a major role in regulating the rate of sucrose synthesis in response to the supply of glucose, and in response to moderate water deficits. In the presence of 300 mM and especially 500 mM mannitol, two- to three-fold higher rates of sucrose synthesis were attained at a given V_{sel}-SPS activity (Fig. 11B, note scale is different from Fig. 11A), showing that an additional factor is stimulating SPS in vivo during extreme water stress. There was a large increase of hexose phosphates in these conditions (see Fig. 4), indicating that SPS is stimulated by increasing availability of substrate and allosteric activator. Figure 11 also reveals that the absolute rates of sucrose synthesis in the discs are about eight times lower than the in vitro V_{sel} SPS activity, which is consistent with the enzyme being substrate-limited in vivo.

Fig. 11A–B. Relation between the unidirectional rate of sucrose synthesis and the activity of SPS measured in the V_{sel} assay with limiting substrates and phosphate. The rates of sucrose synthesis are taken from Table 5, and SPS activity assayed in the presence of limiting substrates and P_i is taken from Fig. 7B, 7E and 7H. Results are shown for discs supplied with 50 mM glucose, with 5 mM glucose and with 20 mM sucrose, 10 mM glucose and 10 mM fructose. **A** Data points corresponding to 0, 100, and 200 mM mannitol. **B** Data points corresponding to 0, 100, 200, 300 and 500 mM mannitol (note the scale on the y-axis is different from that of **A**). The results are shown as the mean ± SE, except where the error bars are smaller than the symbol

Activation of SPS in intact tubers. Investigations of the relation between water relations and sucrose metabolism in water-stressed whole plants is complicated by the problem that the development of water stress in the whole plant will inevitably lead to an inhibition of photosynthesis and a decreased rate of carbon import into the tuber, which will itself have many effects on tuber metabolism. Two other approaches were used to investigate whether water deficits lead to an analogous activation of SPS in intact tubers, which allowed comparison of material with a similar carbohydrate status in the stressed and non-stressed samples.

In one approach, growing tubers were detached from the plant and stored for up to 8 d at 30% humidity, or at 100% humidity to prevent water loss. The fresh weight decreased by 10% over 4 d in the former treatment, whereas it remained unaltered in the latter treatment (Fig. 12A). When SPS activity was measured in the V_{max} assay, no changes were found after detaching the tubers, and there were no differences between the treatments (Fig. 12B). When SPS activity was measured in the V_{sel} assay (with 1 mM Fru6P, 3 mM Glc6P, 6 mM UDPGlc and 5 mM P_i), there was a twofold increase of activity between 1 and 4 d in the tubers stored at 30% humidity (Fig. 12C). This was accompanied by an increase of soluble sugars from 2 d onwards (Fig. 12D). In contrast, when tubers were held at 100% humidity there was no increase of SPS activity in the V_{sel} assay conditions (Fig. 12C) and soluble sugars continued to decline (Fig. 12D).

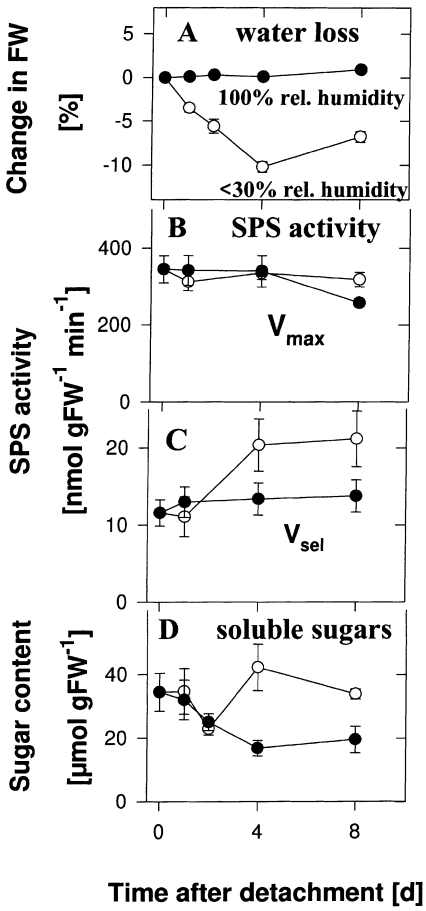


Fig. 12A–D. Sugar metabolism in detached tubers in the presence and absence of water loss. Growing tubers were detached from the plant, and stored at 30% (○) or 100% (●) humidity for up to 8 d. **A** Alteration of fresh weight. **B** SPS activity measured in the V_{max} assay (12 mM Fru6P, 36 mM Glu6P and 6 mM UDPGlc). **C** The V_{sel} assay (1 mM Fru6P, 3 mM Glc6P, 6 mM UDPGlc, and 5 mM Pi) (see legend to Fig. 6 for details). **D** Changes in the sugar content (sucrose plus glucose plus fructose). The results are the mean ± SE of four separate tubers per time point, except where the error bars are smaller than the symbol

In a second approach, a 1-mm-diameter hole was bored through a growing tuber attached to the plant, and filled with water or a 600 mM mannitol solution (Table 6). Three hours later, a concentric 4-mm core was removed and assayed for SPS activity. Similar activities were found in both treatments when the V_{max} assay was used. Activity measured in the V_{sel} assay (with 2 mM Fru6P, 6 mM Glc6P, 6 mM UDPGlc and 5 mM P_i) was very low in the water treatment (6.9% of the V_{max} activity) and increased twofold after perfusion with mannitol (to 11.8% of the V_{max} activity).

Discussion

As water deficit is increased, there is a progressive inhibition of sucrose mobilisation and starch synthesis in discs from growing tubers of *Solanum tuberosum*. This contrasts with the results of Oparka and colleagues, who observed a marked stimulation of label incorporation into starch up to mannitol concentrations of about 300 mM, followed by an inhibition as the water deficit was increased further (Oparka and Wright 1988a,b; Oparka et al. 1990). This difference might be due to use of different cultivars, or to the tubers having experienced different growth conditions prior to the experiment. The following discussion is therefore restricted to the mechanisms whereby water deficit inhibits starch synthesis. We will consider (i) the response to moderate water deficits, and then (ii) the response to more-extreme water stress.

Table 6. Mannitol perfusion in intact growing tubers alters the kinetic properties of SPS. A fine hole (diameter 1 mm) was bored throught growing tubers attached to a 10-week-old plant, filled with 20–30 µl of 0.6 M mannitol, both ends of the hole sealed with petroleum jelly, incubated for 3 h, and a concentric core (diameter 4 mm) removed and assayed for SPS in two different assay conditions (see Table 3). Control samples were taken from tubers treated in an identical manner, except that the borehole was filled with water. The results are mean ± SE (n = 3–4)

	Concentration of mannitol infiltrated into an intact tuber attached to the plant	
	0 mM	600 mM
SPS activity	(nmol gFW^{-1} min^{-1})	
V_{max}	425 ± 18	444 ± 38
V_{sel}	29.4 ± 4.1	55.7 ± 2.5

Moderate water stress leads to a stimulation of SPS, and alters partitioning of carbon in favour of sucrose synthesis. In the range represented by mannitol concentrations of 0–300 mM, there was a relatively small change in fresh weight of the discs. Responses in this region are probably related to changes of turgor (see Oparka and Wright 1988b). Flux measurements reveal that moderate water stress stimulates sucrose synthesis and inhibits sucrose degradation. As a result, the net rate of sucrose mobilisation decreases, and there is a partial inhibition of starch synthesis. These changes are much weaker when the discs are supplied with a high concentration of glucose.

The stimulation of sucrose resynthesis is caused by an activation of SPS (see Figs. 7 and 11A). This activation leads to a change in its kinetic properties and allows higher SPS activity in the presence of limiting substrate concentrations. This explains how sucrose synthesis can be increased by up to twofold, even though the levels of the immediate precursors are unaltered, or even decrease slightly (Fig. 4).

The activation of SPS is probably related to protein phosphorylation. It is blocked by protein phosphatase inhibitors (Table 3), it occurs in the absence of any change in the overall activity of SPS, and it is not accompanied by any change of the SPS immunopositive signal (data not shown). Similar changes of SPS properties can be produced by supplying tuber discs with mannose to sequester P_i (Reimholz et al. 1994), or by feeding high concentrations of glucose (see Fig. 7C,F). This resembles the effects of mannose and increasing rates of photosynthesis on leaf SPS phosphorylation (Huber et al. 1992; Huber and Huber 1996). Post-translational regulation of SPS in the latter cases involves activation by dephosphorylation of ser-158 (Huber and Huber 1996). Further research is needed to establish whether the activation in water stress involves phosphorylation events at the same or a different serine residue, and whether the phosphatase inhibitors are acting directly on SPS, or whether they are acting indirectly by inhibiting a dephosphorylation event that is required in the transduction pathway for water stress.

Potato tuber AGPase is allosterically regulated by 3PGA and P_i (Ballicora et al. 1995). The activation of SPS and stimulation of sucrose synthesis in moderately stressed tuber material leads to a decrease of metabolites, in particular 3PGA. This decrease of 3PGA (between zero and 200 mM mannitol) correlates with a decrease of ADPGlc, and a partial inhibition of starch synthesis (Figs. 9A, 10A). When discs are supplied with a high concentration of glucose, to allow high 3PGA to be maintained in the presence of rapid sucrose synthesis (Figs. 1C, 4A–C, 7C, 8A), there is no decrease of ADPGlc and no inhibition of starch synthesis between 0 and 200 mM mannitol.

We conclude that activation of SPS plays a key role in regulating partitioning in response to moderate water stress in growing tubers (see also Fig. 12). Following activation of SPS and sucrose synthesis, a linking mechanism involving a decrease of 3PGA leads to inhibition of AGPase, and restricts the rate of starch synthesis. This interplay between SPS, 3PGA and AGPase in tubers resembles that previously described in leaves (see *Introduction*).

Water stress also leads to an inhibition of unidirectional sucrose degradation (Fig. 3B, Table 5). We cannot yet explain how sucrose degradation is inhibited by moderate water stress. There is no evidence for a build up of products of the sucrose synthase reaction that could inhibit sucrose degradation. Recent results indicate that sucrose synthase may be regulated by protein phosphorylation (K. Koch, Department of Horticultural Sciences, University of Florida, Fla., USA, S. Huber, personal communication). Water deficits also lead to a decline of sucrose synthase activity within 24 h (data not shown), and it has been reported that abscisic acid leads to a decrease of sucrose synthase transcript (Sowokinos and Varns 1992). More studies are needed to evaluate the contributions of post-translational and transcriptional regulation of sucrose degradation in response to short- and long-term water deficits in tubers.

These changes of partitioning could represent an adaptive response of tuber metabolism to water deficits. If the decreased mobilisation of sucrose leads to a higher concentration of sucrose in the tuber cells, the resulting decrease of the water potential will aid the retention of water and the maintainence of turgor in the growing tuber. Similar changes of SPS activity and sugars also occur in intact tubers (Fig. 12, Table 6). To establish their significance for osmoregulation in growing plants, it will be necessary to develop methods to monitor water movements in and out of tubers on whole plants, and relate these to the changes of metabolism and sugars in the individual growing tubers, and to use reversed genetics to prevent this water-stress induced stimulation of sucrose synthesis.

Severe water stress also decreases starch synthesis by inhibiting reactions subsequent to ADP-glucose pyrophosphorylase. The inhibition of sucrose mobilisation and starch synthesis became stronger at 300–500 mM mannitol and also develops in the presence of high concentrations of glucose, which protect starch synthesis against inhibition at more-moderate water deficits. These high mannitol concentrations lead to large changes of fresh weight, and eventually to plasmolysis of the tuber discs. Alterations of carbohydrate metabolism found in this range could be a response to very low turgor, but could also be the result of alterations of volume or other changes associated with severe water stress. Our results show that additional mechanisms contribute to the changes of partitioning in these conditions.

The stronger inhibition of starch synthesis at 300 and 500 mM mannitol (Figs. 1C, 2C, 3B, 3E, 3H) was accompanied by a large increase of ADPGlc (Fig. 8). This shows that one or more of the reactions subsequent to AGPase is being inhibited. Replotting these results to provide an in vivo ADPGlc saturation curve for starch synthesis indicates that severe water stress decreases the affinity of one or more of the starch synthases for

ADPGlc (compare Figs. 10A and 10B). Our results do not indicate the reasons for this inhibition. It could involve an inhibition of one or more of the starch synthases, or be due to a disturbance of reactions that generate the glucans that act as acceptors for elongation. The appearance of label in the glucan fraction in water-stressed discs (see Figs. 1D, 2D) is also consistent with defects in the processing of the glucans or, alternatively, could reflect increased remobilisation of starch.

The influence of extreme water stress resembles the effect of high temperatures on starch synthesis. These also lead to an accumulation of ADPGlc in tuber discs (data not shown) and an inhibition of starch synthesis (Jenner 1994). In vitro studies have shown that high temperatures reduce the affinity of soluble starch synthase for ADPGlc, by weakening the cooperative binding of the glucan substrate (Jenner et al. 1995).

The increasingly strong inhibition of starch synthesis in severe water stress is accompanied by an accumulation of phosphorylated intermediates (Fig. 4). The rate of sucrose synthesis continues to increase in response to severe water stress above 300 mM mannitol (Figs. 2C, and 3E,H) even though there is no further activation of SPS (Fig. 7, see also Fig. 11B). The rate of sucrose synthesis also increases at high external osmotica in the discs supplied with 50 mM glucose (Fig. 1C), even though there were no changes of SPS activation in response to water stress in this treatment (Fig. 7C). Potato tuber SPS, like the enzyme from leaves, is subject to allosteric activation by Glc6P and inhibition by Pi (Reimholz et al. 1994). The continued increase of sucrose synthesis is probably a consequence of the inhibition of starch synthesis and the resulting accumulation of metabolites under severe water stress. This accumulation of metabolites might also contribute to the inhibition of sucrose degradation.

The relation between the levels of 3PGA and ADPGlc is altered at high mannitol concentrations (Fig. 9B). Lower ADPGlc levels are associated with a given concentration of 3PGA. This might indicate that AGPase is also being inhibited by some unknown mechanism in severly stressed tubers. An alternative explanation is that the decreased protoplasmic volume leads to a corresponding increase in the total concentration of phosphate-containing compounds in the cytosol, in which case a given level of 3PGA would be accompanied by a higher concentration of the inhibitor P_i.

Relation between the level of ADPGlc and the rate of starch synthesis. There has been considerable debate about the contribution of AGPase to the control of starch synthesis. The allosteric properties of the enzyme make it ideally suited to regulate starch synthesis in response to the availability of substrate. Studies of *Chlamydomonas* mutants with altered regulatory properties (Ball et al. 1991) and potato tubers overexpressing native and mutated *E. coli* AGPase (Stark et al. 1992) have demonstrated the significance of the allosteric regulation properties for the rate of starch accumulation in vivo. Studies on *Arabidopsis* mutants with decreased expression of AGPase (Neuhaus and Stitt 1990) have shown that AGPase makes a major contribution to the control of starch synthesis in leaves, with a flux control coefficient of 0.35–0.6, depending on the conditions.

In contrast, studies of pea mutants (Denyer et al. 1990) and potato antisense transformants (Mueller-Roeber et al. 1992) indicate that moderate changes of AGPase expression in developing seeds and tubers do not have a major impact on the amount of starch synthesised, and addition of mannose to tuber discs resulted in only a small stimulation of starch synthesis (Hnilo and Okita 1989). It has been proposed that the high-temperature inhibition of starch synthesis is due to loss of activity (Keeling et al. 1993) or changes in the kinetic properties of soluble starch synthase (Jenner et al. 1995), rather than regulation of AGPase.

This paper presents the first detailed comparison of the relation between the levels of 3PGA, ADPGlc and the rates of starch synthesis in a plant storage organ. There is a very strong correlation between the level of 3PGA and the ADPGlc level (Fig. 9). This provides strong evidence for the importance of the allosteric properties of potato tuber AGPase for the regulation of starch synthesis in vivo. They operate to link the rate of starch synthesis to the availability of carbon, and are also an important component of the regulation circuit that adjusts starch and sucrose metabolism to the water status of the cell.

The in vivo relation between the ADPGlc concentration and the rate of starch synthesis in unstressed or mildly stressed material approximates to a saturation function (Fig. 10A). Obviously, AGPase will not be able to control the rate of starch synthesis in conditions where the ADPGlc concentration is above that needed to saturate the subsequent reactions in the pathway of starch synthesis. When ADPGlc concentrations are sub-saturating for the subsequent reaction, AGPase will contribute to the control of starch synthesis. However, it will only have a high control coefficient when the ADPGlc concentration is strongly sub-saturating.

In unstressed or moderately stressed tuber discs, half-maximal rates of starch synthesis are achieved at below 0.6 nmol ADPGlc $\cdot$ g FW^{-1}, and there is no further stimulation of starch synthesis when ADPGlc rises above 1.8 nmol $\cdot$ g FW^{-1}. The latter high levels were obtained by supplying the discs with very high concentrations of glucose. Clearly, the maximum rate of starch synthesis is limited by the rate at which ADPGlc can be used, rather than by AGPase. In tubers growing in growth cabinets, we have measured ADPGlc levels in different batches of 1.8, 2.2 and 3.2 nmol $\cdot$ g FW^{-1} (Geigenberger et al. 1994, and data not shown). Tubers growing in the glasshouse under low-light conditions contained 0.1–0.8 nmol $\cdot$ g FW^{-1} (data not shown). These results indicate that ADPGlc is sometimes limiting, and is sometimes be near- or completely saturating for starch synthesis in intact tubers. It will be interesting to investigate the reasons for this variability. It is also important to note that the dependence of starch synthesis on the ADPGlc level may be modified under extreme water stress (Fig. 10B) and high temperature (see discussion above).

In conclusion, moderate water stress modifies the fluxes around the sucrose cycle in growing potato tubers, leading to an activation of SPS and sucrose synthesis, and an inhibition of sucrose degradation. The resulting inhibition of net sucrose mobilisation and decline of metabolites, in particular of 3PGA, inhibits AGPase and leads to a partial inhibition of starch synthesis. This regulatory circuit provides one explanation for the widespread presence of SPS and this regulatory cycle of sucrose degradation and resynthesis in growing sink tissues. Studies with transgenic plants are in progress to directly quantify the importance of SPS in controlling this response, and to investigate whether a decreased capacity for sucrose synthesis impairs the ability of growing tubers and other plant organs to cope with water stress. Under more- severe water stress there is also a direct inhibition of the terminal reactions of starch synthesis.

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