Regulation of Protein Degradation and Protease Expression by Mannose in Maize Root Tips. Pi Sequestration by Mannose May Hinder the Study of Its Signaling Properties

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The effects of mannose (Man) and glucose (Glc) on central metabolism, proteinolysis, and expression of the root starvation-induced protease (RSIP; F. James, R. Brouquisse, C. Suire, A. Pradet, P. Raymond [1996] Biochem J 320: 283–292) were investigated in maize (Zea mays L. cv DEA) root tips. Changes in metabolite concentrations (sugars, ester-phosphates, adenine nucleotides, and amino acids) were monitored using in vivo and in vitro 13C- and 31P-NMR spectroscopy, in parallel with the changes in respiration rates, protein contents, proteolytic activities, and RSIP amounts. The inhibition of proteinolysis, the decrease in proteolytic activities, and the repression of RSIP expression triggered by Man, at concentrations usually used to study sugar signaling (2 and 10 mM), were found to be related to a drop of energy metabolism, primarily due to a Man-induced Pi sequestration. However, when supplied at low concentration (2 mM) and with the adequate phosphate concentration (30 mM), energy metabolism was restored and Man repressed proteinolysis similarly to Glc, when provided at the same concentration. These results indicate that Man should be used with caution as a Glc analog to study signalization by sugars in plants because possible signaling effects may be hindered by Pi sequestration.

In higher plants, carbohydrates are not only growth substrates but also developmental regulators that allow plants to cope with environmental conditions. Plant cell metabolism and gene expression may be significantly modulated in response to changing sugar supply (Stitt and Sonnewald, 1995; Koch, 1996). Most frequently, a non-limiting sugar supply triggers the enhancement of carbohydrate-using functions such as respiration, cell cycle, synthesis of polysaccharides, and secondary metabolism products, protein storage, nitrogen assimilation, or Suc metabolism, and the repression of alternative carbon source pathways (starch, lipid, and protein breakdown) and sugar production pathways (photosynthesis). Limiting sugar supply, conversely, induces alternative carbon source pathways and photosynthesis, and represses sugar-using functions (for reviews, see Koch, 1996; Smeekens, 1998; Sheen et al., 1999).

The mechanisms used by living cells to sense sugars have been extensively studied in yeast, animal, and plant cells, and there is increasing evidence that sugar-sensing mechanisms appear to be conserved in eukaryotic cells (Jang and Sheen, 1997; Smeekens, 1998; Halford et al., 1999; Johnston, 1999). The use of sugars, non-metabolizable sugar analogs, or metabolic intermediates suggests several potential locations for signal input into the sugar-sensing system of the cell. Thus, evidence for the occurrence of several sensing points such as hexose or Suc transport into the cytosol, or hexose phosphorylation by hexokinase, was obtained in plant cells (for review, see Jang and Sheen, 1997; Jang et al., 1997; Smeekens, 1998). Other sensing points downstream of hexokinase, either related to glycerol or acetate metabolism (Sheen, 1990; Graham et al., 1992), or linked to the respiratory substrate supply to mitochondria (Aubert et al., 1996), were also postulated (Jang and Sheen, 1997; Halford et al., 1999).

Among the sugar analogs, Man has been widely used to investigate sugar signaling. In many plants, Man, like 2-deoxy-Glc (2DOG) or 2-amino-2-deoxy-Glc, is actively transported into the cells and phosphorylated by hexokinase but is then metabolized slowly. Thus, contrary to 3-O-methyl-Glc or 6-deoxy-

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Glc, which are not phosphorylated, Man is considered to mimic Glc signaling in the regulation of photosynthetic and glyoxylate cycle genes (Graham et al., 1994; Jang and Sheen, 1994). In celery, a mannotol metabolizing plant, Man was also shown to repress mannotol dehydrogenase activity (Prata et al., 1997), and in other species it was found to inhibit the germination (Matheson and Myers, 1998; Pego et al., 1999). Such results led the authors to conclude that hexose phosphate metabolism is not required for sugar sensing, and that hexokinase could be a hexose sensor involved in sugar signaling (Graham et al., 1994; Jang and Sheen, 1994; Pego et al., 1999; Sheen et al., 1999).

However, Man was often described to be toxic (Herold and Lewis, 1977). At the plant or tissue level, Man was shown to inhibit the growth of shoots, roots, stems, and pollen tubes (Herold and Lewis, 1977), inhibit the germination of pollen and seeds (Nygaaard, 1971; Matheson and Myers, 1998; Pego et al., 1999), and to induce leaf wilting (Herold and Lewis, 1977). At the cell level, Man was shown to unbalance Glc and ion uptake (Herold and Lewis, 1977), respiration, ATP synthesis (Garrard and Humphreys, 1969), photosynthesis (Harris et al., 1983, 1986), starch synthesis, and degradation (Herold and Lewis, 1977) and to induce cell apoptosis (Stein and Hansen, 1999). The primary cause of most of the inhibitory effects of Man is the accumulation of Man-6-P (M6P) because of the absence or low activity of Man phosphate isomerase (Herold and Lewis, 1977). This accumulation results in a strong Pi sequestration, which leads to a decreased synthesis of ATP and a subsequent imbalance of metabolism (Herold and Lewis, 1977). 2DOG has also been shown to sequester Pi as 2DOG-6-P and to trigger similar effects as Man (Roberts et al., 1980; Klein and Stitt, 1998). In some studies, Man or 2DOG have been used to induce phosphate deprivation states (Sieg1 and Stitt, 1990; Van Quy and Champigny, 1992; Farrar et al., 1995). On the other hand, Man and M6P have been shown to inhibit various enzymes of carbohydrate metabolism such as gluco- and fructokinase, Glc phosphate isomerase, phosphofructo kinase, Fru bisphosphatase, aldolase, Glc-6-P dehydrogenase, or phosphoglucomutase (Herold and Lewis, 1977). In conclusion, it is necessary to control Man toxicity for cell metabolism when using Man as a Glc analog to investigate sugar signaling in plants.

In plants, under sugar deprivation, proteins have been shown to be degraded, thus supplying carbon skeletons to respiration and biosynthetic processes (James, 1953; Thomas, 1978; Journet et al., 1986; Baysdorfer et al., 1988; Peoples and Dalling, 1988; King et al., 1990; Brouquisse et al., 1991, 1992; Tassi et al., 1992). We have previously shown that in maize (Zea mays L. cv DEA) root tips submitted to sugar deprivation protein degradation is related to the induction of a vacuolar Ser endopeptidase, named root starvation-induced protease (RSIP), which accounts for approximately 80% of the total endopeptidase activity measured in vitro in starved root tip extracts (James et al., 1996; Brouquisse et al., 1998). A Cys protease was also shown to be induced in sugar-starved maize root tips (Chevalier et al., 1995). The induction of proteases may be reversed by the addition of sugars in the incubation medium (James et al., 1993; Chevalier et al., 1995). This suggests that the expression of these proteins is directly related to the supply of sugars to the cell.

In the present work, we investigated the effects of Man on central metabolism, proteolysis, and RSIP expression in maize root tips. Using in vivo and in vitro $^{13}$C- and $^{31}$P-NMR spectroscopy, we monitored the variations in metabolite concentrations (sugars, ester-phosphates, adenine nucleotides, and amino acids). In parallel, we followed the changes in respiration rates, protein contents, proteolytic activities, and RSIP amounts. We found that the inhibition of proteolysis and the repression of protease triggered by Man at concentrations usually used to study sugar signaling were related to a drop of energy metabolism, primarily due to a Man-induced Pi sequestration. However, when supplied at low concentration and with the adequate phosphate concentration, Man also repressed proteolysis similarly to Glc when provided at the same concentration. Sugar signaling or Pi sequestration effects of Man on sugar-regulated proteolysis are discussed in relation to these results.

**RESULTS**

Figure 1 summarizes the changes of several parameters related to protein degradation in response to sugar starvation in excised maize root tips. As already reported (Saglio and Pradet, 1980; Brouquisse et al., 1991), the removal of sugar supply immediately triggered a sharp decline in sugar content that decreased to 15% of its initial value and then remained steady for up to 4 d of starvation (Fig. 1). In

![Figure 1](www.plantphysiol.org)
the same time protein content decreased continuously. During the first 4 d, protein mobilization was related to a transient increase in free amino acid content, mainly Asn, and to the induction of endopeptidase activities (Brouquisse et al., 1992; James et al., 1993, 1996; Chevalier et al., 1995). Beyond 4 d, the starvation becomes irreversible (Brouquisse et al., 1991) as metabolite contents and enzyme activities decreased. To investigate the effects of Man and Glc on metabolism, proteolysis, and RSIP expression, a 48-h incubation period was chosen because the changes in enzymic activities and metabolite contents are clearly marked and the starvation remained fully reversible.

**Effects of Man and Glc on Protein Content and Proteolytic Activities**

Figure 2 reports the changes in total protein content, endopeptidase activities, and RSIP expression that occurred in the root tips after a 48-h incubation period in the presence of increasing concentrations of either Glc or Man in comparison with control (Ctrl) and starvation (Star) situations. Increasing concentrations of Glc led to increased protein contents (from 80–115 μg tip⁻¹, Fig. 2A) and decreased endopeptidase activities (from 32–11 ng min⁻¹ tip⁻¹) and RSIP amounts (Fig. 2, B and C). On the contrary, with 10 to 200 mM Man, protein contents, endopeptidase activities, and RSIP expression did not change significantly compared with the control or to 200 mM Glc. In the presence of 2 mM Man, starvation symptoms appeared with a moderate decrease in protein content and a small increase in both endopeptidase activities and RSIP expression (Fig. 2). Thus, in terms of protein degradation and protease expression, 10 mM Man, which is in the range of concentrations commonly used in experimental models to investigate the sugar effect of Man (Graham et al., 1994; Jang and Sheen, 1994; Klein and Stitt, 1998; Pego et al., 1999), caused the same effects as 200 mM Glc. The question raised by these results was as to whether the inhibition of proteolysis by low Man concentrations was due to a strong sugar-effect of Man itself or to secondary effects on cell metabolism. Therefore, we investigated the changes in respiration rate and in various metabolites either involved in the primary metabolism or used as markers of cell component degradation in maize root tips supplied with either 10 mM Man or 200 mM Glc.

**Effects of Man on Maize Root Tip Metabolism: ¹³C and ³¹P in Vivo NMR Study**

In vivo NMR spectroscopy is a well-established technique to follow the concentration of abundant mobile metabolites within a sample as a function of time. Figure 3 displays typical ³¹P and ¹³C spectra obtained with living maize root tips during transition substrates experiments. In the present experiments, ¹³C-NMR was used to monitor Asn, a transient marker of protein degradation (Brouquisse et al., 1992), whereas ³¹P-NMR was used to analyze the most abundant phosphorylated compounds, such as hexose-phosphates, inorganic phosphate (Pi), ATP, and phosphoryl choline (P-chol), which is a marker of membrane degradation (Roby et al., 1987).

Transition from 200 mM Glc to 10 mM Man immediately led to an important reduction in respiration rate and ATP content in maize root tips (Fig. 4A). In the same time, M6P and Man-1-P (M1P) markedly accumulated, whereas cytoplasmic and vacuolar Pi (cyt-Pi and vac-Pi, respectively) contents dropped (Fig. 4B). UDP-Man was also shown to accumulate in the root tips (Fig. 4B, insert). Ten to 15 h after the transition, respiration rate and metabolite contents reached a quasi-steady state and finally evolved moderately up to 48 h of incubation (Fig. 4, A and B). The measurement of [¹⁴C]CO₂ production, after [¹⁴C]Man labeling, showed that Man was poorly metabolized by maize root tips and contributed for less...
than 100 pmol O₂ min⁻¹ tip⁻¹ to the respiration (i.e. 7% maximum of the total O₂ consumption) after 48 h of incubation (Fig. 4A, insert). Moreover, Man phosphate isomerase (MPI) activity was non-detectable (below 20 pmol min⁻¹ tip⁻¹) in Man-treated root tips as well as in control root tips. It may be noted that, after 48 h of incubation on 10 mM Man, root tip growth was totally stopped (data not shown), although no symptom of massive protein and lipid degradation occurred, since Asn remained steady and P-chol decreased to the same extent as ATP and cyt-Pi (Fig. 4A).

Taken together, these data suggest that Man was phosphorylated at the expense of cellular Pi and of other phosphorylated compounds, such as P-chol. As already reported (Herold and Lewis, 1977; Harris et al., 1986; Loughman et al., 1989), the immobilization of cellular Pi into M₆P significantly reduces Pi and ATP turnover, and thus triggers a slowing down of the cell metabolism. In the present work, the transition from 200 mM Glc to 10 mM Man triggered an immediate reduction in global protein turnover. This transition resulted in a significant change in the net protein balance (NPB), going from net protein synthesis (PS) to net protein degradation (PD; Fig. 5). Then, between 6 and 24 h of incubation, both PS and PD transiently increased. This temporary increase in both processes was attributed to an acclimation process by analogy to previous observations (Brouquisse et al., 1992). From Figure 5, total PS and PD values calculated for 2 d were respectively +47.9 and −63.7 µg protein tip⁻¹. The resulting NPB was −15.8 µg protein tip⁻¹, which fits the data presented in Figure 2A (Man, 10 mM).

Contrary to Man, a 48-h incubation with 200 mM Glc did not cause significant changes in metabolite content. As a consequence of Glc import and metabolism, respiration rate and G6P content moderately increased (by 10% within 48 h), and vac-Pi slightly decreased, whereas cellular phosphate, P-chol, and Asn remained unchanged (data not shown). In these conditions, protein turnover remained steady for 48 h, and root tip growth occurred.

Figure 3. In vivo ³¹P- and ¹³C-NMR study of metabolic transitions induced in excised maize root tips. Proton-decoupled ³¹P-NMR (A) and ¹³C-NMR (B) spectra of 2,500 excised root tips in normoxia at 21°C, perifused with a nutritive medium at pH 6.5 containing successively 200 mM Glc (control), no carbon substrate (starvation), or 10 mM Man. For each line, the time indicates the duration of the treatment up to the end of the spectrum. ³¹P acquisition parameters (A), 7,150-Hz frequency window, 30-µs pulse length (approximately 45° pulse angle), 0.6-s repetition period, spectrum obtained in 1 h. Lorentz-Gauss apodization and zero filling. ¹³C-acquisition parameters (B), 22,700-Hz frequency window, 30-µs pulse length (approximately 30° pulse angle), 2.0-s repetition period, spectrum obtained in 2 h. Exponential apodization and zero filling. Abbreviations: Pi, inorganic phosphate; G6P, Glc-6-P; M₆P, Man-6-P; M₁P, Man-1-P; P-eth, phosphorylethanolamine; P-chol, phosphorylcholine; F6P, Fru-6-P; UDPG, UDP-Glc; P₇c, cytoplasmic Pi; Pᵥ, vacuolar Pi; N, Asn.
The capacity of Man and Glc to reverse the effects of a preliminary sugar starvation and to restore root tip growth was tested in a second set of experiments. Figure 6 shows the changes in metabolism occurring in maize root tips that are successively fed with Glc, carbon-starved for 48 h, and fed again with 10 mM Man. As expected from previous work (Journet et al., 1986; Roby et al., 1987; Brouquisse et al., 1991), carbon starvation triggered a rapid fall in the respiration rate and a decrease in soluble sugars (Fig. 3), G6P, and ATP contents (Fig. 6, A and B). The accumulation of P-chol and Asn (Figs. 3 and 6A), the decrease in protein content, and the increase in proteolytic activity and RSIP amount (Fig. 6C) indicated that lipids and proteins were degraded. In this situation, Pi readily accumulated into the vacuole, whereas cyt-Pi remained more or less steady (Fig. 6B). Transition from starvation to 10 mM Man changed cellular metabolism markedly. Within 15 h, M6P and M1P accumulated (up to 11 and 1.2 nmol tip⁻¹, respectively), whereas cyt-Pi, vac-Pi, P-chol, and ATP dropped dramatically (Figs. 3 and 6, A and B). The first result is that Man trapped the cellular Pi as M6P, as already observed in Figure 4. The second one is that Pi may be rapidly exported from the vacuole to the cytoplasm in response to a strong demand of Pi in agreement with Plaxton (1998). The third one is that the P-chol, accumulated in response to carbon starvation, was promptly dephosphorylated when the Pi demand increased. Furthermore, the addition of Man clearly stopped protease induction, proteolysis, and Asn accumulation (Figs. 3 and 6, A and C). In contrast, when 200 mM Glc was added to the incubation medium after 2 d of carbon starvation, a progressive return to the initial conditions was observed with an increase in respiration rate, in the content of esterphosphate and ATP, and a progressive decline in vac-Pi, Asn, and P-chol (data not shown). Endopeptidase activities and RSIP amount, accordingly, declined, whereas protein content increased (Fig. 7).

Taken together, these data showed that, in maize root tips, Man was poorly metabolized and could not be substituted for Glc as a growth or recovery substrate. Man accumulated as M6P, probably because of the lack of phospho-Man isomerase activity, and thus triggered a phosphate deprivation state in the cell. This resulted in a decline in respiration, a subsequent decrease in ATP, and a more or less marked inhibition of energy consuming processes, including PS and PD. Therefore, we examined whether Pi sequestration by Man could be overcome by the addition of exogenous Pi.

Figure 4. Metabolic response of excised maize root tips to the Glc to Man transition. Time course of the respiration rate measured online during the NMR experiment, and of the concentrations of the more abundant metabolites detected in vivo. Excized root tips were fed for 4 h with 200 mM Glc and then with 10 mM Man for up to 48 h. A, Respiration rate (●), ATP (■), P-choline (○), and Asn (▲) contents. Inset, The amount of Man consumed by respiration (●) and the percentage of Man contribution to total O₂ consumption (■) were measured after incubation of the root tips in the presence of [¹⁴C]Man as described in “Materials and Methods.” B, Cyt-Pi (●), vac-Pi (○), G6P (■), M6P (▲), and M6P+Glyc3P (□). Inset, UDP-Man (▲). This is a representative experiment.
Effects of Man and Phosphate on Energy Metabolism and Proteolysis

Maize root tips were incubated for 48 h in the presence of either 2 or 10 mM Man and increasing concentrations of Pi (0, 0.25, 10, 30, and 100 mM). As shown in Table I, total adenine nucleotide (AdN) content, ATP/ADP ratio, and adenylate energy charge (AEC) value were markedly reduced in Man-treated root tips compared with control or starved root tips. These results confirmed that a major effect of Man in maize root tips is the impairment of energy metabolism via Pi deprivation. Only in the presence of 2 mM Man and 30 mM Pi did the values of ATP/ADP ratio and AEC remain close to those obtained in control, starved, or 2 mM Glc-treated root tips (Table I). PD, endopeptidase activities, and RSIP expression similarly were shown to depend on Pi availability and the energy status of the cell (Fig. 8). In the presence of 10 mM Man, neither proteolysis nor protease induction significantly occurred as ATP/ADP ratio and AEC value remained low. With 2 mM Man, proteolysis, endopeptidase activities, and RSIP expression increased (Fig. 8), together with ATP/ADP ratio and AEC value (Table I), as Pi supply increased. In 2 mM Man-/30 mM Pi-treated root tips, protein levels, endopeptidase activities, and RSIP expression were found to be similar to those obtained in 2 mM Glc-treated root tips (Figs. 2 and 8). The recovery of energy metabolism and proteolytic processes in Pi supplied root tips cannot be ascribed to the induction of Man metabolism by Pi since phospho-Man isomer-

ase activity remained non-detectable (below 20 pmol min$^{-1}$ tip$^{-1}$) in all the root tip samples (data not shown). It may be noted that when supplied at high concentration (100 mM), Pi became toxic for maize root tips and leads to a dramatic decrease in AdN and protein contents, and in endopeptidase activities (Table I; Fig. 8). These data show that, when Man is used at low concentration together with adequate Pi concentration: (a) the toxic effect of Man on energy metabolism may be overcome, and (b) Man apparently regulates proteolysis via a sugar-effect similar to that of Glc.

Effects of Low Man and High Pi Concentrations on Maize Root Tip Metabolism: $^{13}$C and $^{31}$P in Vitro NMR Study

In vitro $^{13}$C- and $^{31}$P-NMR spectroscopy was used together with respiration measurements to analyze the molecular composition and the respiration rate of maize root tips incubated for 48 h with either 2 mM Glc/30 mM Pi or 2 mM Man/30 mM Pi, in comparison with control or carbon-starved root tips. As expected from above data (Figs. 3 and 6), carbon starvation was characterized by a marked decrease in the respiration rate (from 3.27–1.17 nmol O$_2$ min$^{-1}$ tip$^{-1}$) and in the level of soluble sugars and sugar phosphates (Fig. 9; Table II). Conversely P-chol, P-eth, and Asn clearly increased as well as free Pi. Root tips incubated with 2 mM Glc/30 mM Pi also exhibited starvation symptoms, although less marked than in starved root tips with a drop in soluble sugars, G6P, and UDP-GLC, and an increase in P-chol, P-eth, and Asn (Fig. 9; Table II). However, respiration only decreased by 25% instead of 64% in starved root tips. This means that 2 mM Glc was actively taken up and metabolized (Table II), although insufficient to sustain optimal respiration rate. After 48 h in the presence of 2 mM manose/30 mM Pi, the respiration of the root tips did not differ significantly from that of carbon-starved root tips, confirming that Man, which phosphorylated products (M6P, M1P, and UDP-Man) accumulated, was poorly metabolized (Table II). Contrary to starved root tips, Man-treated root tips were not totally depleted in Suc and Glc. This may be explained by the fact that Man competes with Glc for uptake and phosphorylation by hexokinase. And last, 2 mM Man- and 2 mM Glc-treated root tips exhibit similar increase in P-chol, P-eth, and Asn (Fig. 9; Table II). In agreement with the data presented in the Figure 8, these results show that, provided that Pi requirements are fulfilled, 2 mM Glc or 2 mM Man triggered the same response in terms of proteolysis, and possibly lipolysis, set up.

DISCUSSION

In the present work, we show that, in excised maize root tips, PD, endopeptidase activities, and RSIP ex-
pression were inhibited by Glc in a concentration-dependent manner (Fig. 2). At a relatively low concentration (10 mM) Man also inhibits proteolysis and protease expression as strongly as 200 mM Glc. Ten to 20 mM Man similarly were shown to severely repress the gene expression of isocitrate lyase and malate synthase genes in cucumber cells (Graham et al., 1994), chlorophyll a/b-binding protein (cab) in maize protoplasts (Jang and Sheen, 1994), and Rubisco small subunit (rbcS) in Chenopodium rubrum (Klein and Stitt, 1998). Pego et al. (1999) recently showed that 7.5 to 15 mM Man were sufficient to abolish Arabidopsis seed germination, whereas no inhibition was observed with 10 mM Glc or Fru. These data raised the question why Man, at concentrations lower than that which trigger apoptosis (Stein and Hansen, 1999), inhibits gene expression and biological processes (in the present work, proteolysis, and RSIP expression) more strongly than Glc? Are these inhibitions due to a "strong" sugar effect, mediated or not by hexokinase, or to secondary effects due to Man toxicity?

Present data show that, in maize root tips, Man is phosphorylated into M6P at the expense of cytoplasmic and vacuolar Pi and of other phosphorylated compounds but poorly supplies respiration with carbon skeletons (Figs. 3, 4, and 6; Table I). The sequestration of Pi under M6P severely impaired primary metabolism with a dramatic drop in glycolytic intermediates, respiration rate, and adenine nucleotide content (Figs. 4 and 6; Tables I and II). Some of these effects have been already described to occur in Man-treated tissues (Kime et al., 1982; Loughman et al., 1989; Siegl and Stitt, 1990; Van Quy and Champigny, 1992; Farrar et al., 1995; Klein and Stitt, 1998) and may be clearly associated with Pi starvation symptoms (Plaxton, 1998). One of the direct consequences is that both PS and PD are affected by Pi sequestration (Figs. 2, 5, and 8). Thus, the low PD, endopeptidase activities, and RSIP expression in 10 mM Man incubated root tips (Figs. 2, 4, and 5) are obviously not due to a sugar repression mechanism but rather to an inhibition of the metabolism triggered by Pi sequestration. This explanation is supported by refeeding experiments that show that, whereas PD and RSIP expression may be reversed by 200 mM Glc addition (Fig. 7), no reversion occurs after 10 mM Man addition (Fig. 6). Rather, protein content, endopeptidase activities, and RSIP level remain steady, and energy metabolism drops (Fig. 6). Furthermore, it should be kept in mind that Man and M6P are known to inhibit some enzymic activities related to sugar metabolism (Herold and Lewis, 1977; Matheson and Myers, 1998) and that beside its effect on energy metabolism, decreased Pi availability may also impaired enzyme regulation or the operation of signal transduction pathways (Siegl and Stitt, 1990; Van Quy and Champigny, 1992; Sugden et al., 1999).
The reversion of Man effects on energy metabolism and proteolysis by the addition of external Pi (Table I; Fig. 8) raises several comments: First, at least with maize roots, Man concentration should be sufficiently low (i.e. 2 mM) so that its toxic effects can be reversed by Pi addition; ATP/ADP ratio and AEC could not be restored by Pi addition with 10 mM Man (Table I). Second, to reverse Man effects, Pi should be supplied at high concentration compared with Man. In our experiments ATP/ADP ratio and AEC were close to the control values with a [Pi]/[Man] ratio = 15. This probably explains why, in previous studies, Man effects were not reversed when Pi was added at the same concentration as Man (Graham et al., 1994; Jang and Sheen, 1994; Matheson and Myers, 1998). Third, with 2 or 10 mM Man, an excess of Pi (i.e. 100 mM) turns out to be toxic for maize roots and results in a loss of nucleotide and protein contents without protease induction (Table I; Fig. 8). Cytoplasmic Pi concentration of Pi-sufficient plant cells usually ranges from 5 to 15 mM (Theodorou and Plaxton, 1993), but an excess of Pi is known to inhibit the expression of sugar-inducible genes (Sadka et al., 1994), the activities of enzymes like phosphatases (Duff et al., 1994), and general plant growth (Rorison, 1969). Thus, it is likely that the supply of increasing amounts of Pi allows the recovery of Pi turnover, energy metabolism, and, in the present work, proteolytic processes, up to the point where Pi accumulates in excess and becomes toxic by itself. Such an excess of Pi could explain the inhibition of germination of Arabidopsis seeds treated with 7.5 mM Man and 75 mM Pi (Pego et al., 1999) or the decrease in metabolite contents observed in Chenopodium cells treated with 0.2 mM 2DOG and 30 mM Pi (Klein and Stitt, 1998). Fourth, proteolytic processes appear to be controlled by the energy status of the cell, that is ATP/ADP ratio and AEC value, rather than absolute ATP or AdN content.

Table 1. Effect of varying mannose and phosphate supply on adenine nucleotide contents, ATP/ADP ratio, and AEC in maize root tips

<table>
<thead>
<tr>
<th></th>
<th>ATP (nmol/tip)</th>
<th>ADP (nmol/tip)</th>
<th>AMP (nmol/tip)</th>
<th>Σ AdN (nmol/tip)</th>
<th>ATP/ADP</th>
<th>AEC</th>
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<tr>
<td>Control</td>
<td>1,836</td>
<td>360</td>
<td>&lt;2</td>
<td>2,197</td>
<td>5.1</td>
<td>0.91</td>
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<tr>
<td>Starvation</td>
<td>1,147</td>
<td>225</td>
<td>&lt;2</td>
<td>1,373</td>
<td>5.0</td>
<td>0.91</td>
</tr>
<tr>
<td>Glu 2 mM</td>
<td>1,205</td>
<td>241</td>
<td>&lt;2</td>
<td>1,447</td>
<td>4.9</td>
<td>0.90</td>
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<tr>
<td>Man 2 mM</td>
<td></td>
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<td></td>
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<tr>
<td>Pi 0 mM</td>
<td>313</td>
<td>165</td>
<td>11</td>
<td>489</td>
<td>1.9</td>
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</tr>
<tr>
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<td>228</td>
<td>22</td>
<td>725</td>
<td>2.1</td>
<td>0.81</td>
</tr>
<tr>
<td>10 mM</td>
<td>700</td>
<td>188</td>
<td>5.7</td>
<td>894</td>
<td>3.7</td>
<td>0.89</td>
</tr>
<tr>
<td>30 mM</td>
<td>762</td>
<td>141</td>
<td>&lt;2</td>
<td>904</td>
<td>5.4</td>
<td>0.92</td>
</tr>
<tr>
<td>100 mM</td>
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<td>2.1</td>
<td>27</td>
<td>1.1</td>
<td>0.69</td>
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<tr>
<td>Man 10 mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pi 0 mM</td>
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<td>275</td>
<td>74</td>
<td>716</td>
<td>1.3</td>
<td>0.70</td>
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<tr>
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<td>61</td>
<td>691</td>
<td>1.6</td>
<td>0.74</td>
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<tr>
<td>10 mM</td>
<td>447</td>
<td>236</td>
<td>77</td>
<td>760</td>
<td>1.9</td>
<td>0.74</td>
</tr>
<tr>
<td>30 mM</td>
<td>446</td>
<td>215</td>
<td>47</td>
<td>708</td>
<td>2.1</td>
<td>0.78</td>
</tr>
<tr>
<td>100 mM</td>
<td>107</td>
<td>35</td>
<td>5.3</td>
<td>147</td>
<td>3.1</td>
<td>0.85</td>
</tr>
</tbody>
</table>
Indeed, after Pi addition, PD and RSIP expression are progressively restored when ATP/ADP ratio and AEC return close to control values (Fig. 8; Table I). Thus, 2 mM Glc/0.25 mM Pi- and 2 mM Man/30 mM Pi-treated root tips exhibit similar ATP/ADP ratios, AEC values, protein contents, endopeptidase activities, and RSIP expression (Figs. 2 and 8; Table I), whereas they clearly differed in ATP and AdN contents (Table I). These data do not support the hypothesis that the slowing down of the metabolism induced by Man or 2DOG would be due to the decrease in ATP content (Garrard and Humphreys, 1969; Harris et al., 1986).

Figure 8. Effects of varying Man and phosphate supply on protein content (A), proteolytic activities (B), and RSIP expression (C) in maize root tips. Excised root tips were incubated for 48 h with either 2 or 10 mM Man, in the presence of various concentrations (0, 0.25, 10, 30, or 100 mM) of Pi, and analyzed as described in “Materials and Methods.” Star, 48-h-starved root tips. Ctrl, T0 control root tips. Total proteins from 1 root tip equivalent (between 144 and 43 μg of protein according to Fig. 8A) were loaded in each lane for western-blot RSIP analysis. Data are the mean of three independent experiments. SD are below 15%.

Finally, the question is raised as to whether Man may be used efficiently to investigate sugar signaling in plant systems. With the excised maize root tips model, we found that 2 mM Man and 2 mM Glc treatments produced the same effects in terms of PD, endopeptidase activities, RSIP expression, and possibly lipid degradation, as far as Pi requirement is fulfilled (Figs. 8 and 9; Tables I and II). Considering the poor metabolization of Man, downstream M6P, and the low respiration rate of 2 mM Man-treated root tips compared with that of 2 mM Glc-treated ones (Fig. 6; Table II), it is tempting to conclude that, with 2 mM Man/30 mM Pi, Man mimics Glc signaling effects and that hexokinase could be involved in the transduction of sugar signal. It appears that the presence or absence of the MPI in the tissues is a key point to consider when using Man as a Glc analog in sugar-sensing studies. When MPI is present, such as in red alga *Galdieria sulfuraria* (Heilmann et al., 1997), in legume seeds (McCleary and Matheson, 1976), or probably in *Vitis vinif-*
Table II. Effects of low Man and high phosphate supply on respiration rate and Pi, ester-phosphates, sugars, and amino acid contents in maize root tips

Excised root tips (1,900) were incubated either for 4 h in a medium containing 200 mM Glc (control), or for 48 h in a medium containing no carbon substrate (starvation), 2 mM Glc/30 mM Pi, or 2 mM Man/30 mM Pi. Tissue extracts, prepared as described in “Materials and Methods,” were analyzed at 21°C with a Bruker AMX 400 WB spectrometer. A concentric capillary containing 13.75 mM TMSP and 0.975 mM methyl diphosphonate in water provided an external reference for $^{31}$P chemical shifts and also intensities, respectively. The molecular concentrations were determined from the resonance intensities of the relaxed spectra taken as peak areas. Peaks of interest and references were integrated and the calibrate reference allowed direct calculation of the molecular concentrations in the extract, and then within one root tip. Pi, inorganic phosphate; G6P, Glc-6-P; M6P, Man-6-P; M1P, Man-1-P; P-eth, phosphorylethanolamine; P-chol, phosphorylcholine; PEP, phosphoenolpyruvate; UDPG, UDP-Glc; UDPM, UDP-Man, so are below 20%.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>$\delta$ ppm</th>
<th>Control</th>
<th>Starvation</th>
<th>Glc 2 mM Pi 30 mM</th>
<th>Man 2 mM Pi 30 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiration</td>
<td>2.3</td>
<td>15.7</td>
<td>41</td>
<td>260</td>
<td>170</td>
</tr>
<tr>
<td>$^{31}$P-NMR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pi</td>
<td>-12.56 and -12.69</td>
<td>1.67</td>
<td>0.72</td>
<td>0.52</td>
<td>0.34</td>
</tr>
<tr>
<td>G6P</td>
<td>-13.58 and -13.70</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>M6P</td>
<td>-13.58 and -13.70</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>M1P</td>
<td>-13.58 and -13.70</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>P-Chol</td>
<td>0.02</td>
<td>1.37</td>
<td>0.13</td>
<td>0.15</td>
<td>0.02</td>
</tr>
<tr>
<td>P-Eth</td>
<td>4.36 and 4.32</td>
<td>5.2</td>
<td>1.1</td>
<td>1.4</td>
<td>1.1</td>
</tr>
<tr>
<td>PEP</td>
<td>2.3</td>
<td>15.7</td>
<td>41</td>
<td>260</td>
<td>170</td>
</tr>
<tr>
<td>UDPG</td>
<td>3.3</td>
<td>1.75</td>
<td>0.78</td>
<td>6.9</td>
<td>6.9</td>
</tr>
<tr>
<td>UDPM</td>
<td>4.28</td>
<td>0.45</td>
<td>0.15</td>
<td>0.35</td>
<td>6.8</td>
</tr>
<tr>
<td>$^{13}$C-NMR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suc</td>
<td>104.40</td>
<td>93</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>29</td>
</tr>
<tr>
<td>Glc</td>
<td>96.70</td>
<td>74</td>
<td>&lt;1</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Man</td>
<td>95.04</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>12.5</td>
</tr>
<tr>
<td>Asp</td>
<td>35.60</td>
<td>3</td>
<td>175</td>
<td>190</td>
<td>120</td>
</tr>
</tbody>
</table>

Rea cells (Vitrac et al., 2000), Man may be used as a growth carbon substrate, but no conclusion may be drawn regarding the role of hexokinase in sugar sensing (Vitrac et al., 2000). With experimental materials where MPI is absent or present at a low level, care should be taken to distinguish Man toxicity effects from Man sugar signaling properties.

MATERIALS AND METHODS

Plant Materials and Incubation Conditions

Maize (Zea mays L. cv DEA, from Pioneer France Maïs, Toulouse, France) seeds were soaked for 3 h in water and germinated for 3 d at 25°C in the dark on layers of wet filter paper as described in Saglio and Pradet (1980). Three-millimeter-long primary root tips were excised and incubated at 21°C to 23°C in a liquid medium flushed with a 50% O$_2$ + 50% N$_2$ gas mixture. Basic incubation medium, containing 0.25 mM Pi, was the mineral medium described by Saglio and Pradet (1980), plus 10 mM MES [2-(N-morpholino)ethanesulfonic acid]-KOH (pH 6.0) and was designated as medium A. Except for adenine nucleotide measurements, root tips were re-excised to 3 mm after 200 mM Glc treatment. For each experiment, 500 to 3,000 root tips were first excised, incubated, and rinsed 2 times in medium A containing 200 mM Glc at ambient temperature. At the end of this preparation period, of approximately 4 h from the first excision, the sample constituted the T0-sample used as the control reference. Then, aliquots of the sample were either perifused in the NMR tube for in vivo measurements or incubated in flasks or syringes for biochemical measurements or acid extracts. Depending on the experiments, the incubation medium was supplemented with an antibiotic-antimycotic mixture (ref A-7292, Sigma, St. Louis) in the presence or absence of potassium phosphate and of Glc or Man. At different times, root tip samples were quickly harvested, frozen in liquid nitrogen, and stored at −193 or −80°C until further analysis.

In Vivo Protein Radiolabeling and Protein Turnover Measurement

PS and PD were determined by pulse-chase experiments after in vivo labeling of the proteins with either $[^{35}$S]Met or $[^{14}$C]Leu. One thousand excised maize root tips were incubated as described above in 250 mL of medium A supplemented with 200 mM Glc, antibiotics-antimycotics, and either 5 mM $[^{35}$S]Met (30 MBq/mmol) or 5 mM $[^{14}$C]Leu (1.48 MBq/mmol). After 24 h, root tips were abundantly rinsed with medium A supplemented with 5 mM cold Met or Leu and then incubated for 48 h in medium A containing 5 mM cold Met or Leu, antibiotics-antimycotics, in the presence of 10 mM Man or 200 mM Glc. Samples of 25 root tips were harvested every 6 h, rinsed with distilled water, and frozen in liquid N$_2$. Proteins of the root tips were
extracted in a glass Potter with 500 μL of an extraction medium containing 50 mM Tris, pH 7.4, 25 mM NaCl, 0.1 (p/v) Triton X-100, and 5 mM β-mercaptoethanol. The crude extract was centrifuged at 25,000 g for 12 min, and the resulting supernatant was supplemented to 1 mL with water. It constituted the crude extract. Five hundred microliters of the crude extract were then desalted through an Econo-Pac DG column (Bio-Rad Laboratories, Hercules, CA) equilibrated with 50 mM Tris, pH 7.4, and 25 mM NaCl. Aliquots of the crude extract were used to determine total soluble proteins (P) and aliquots of the desalted extracts were used to determine the specific radioactivity and the total radioactivity (RA) of the soluble proteins. Radioactivity was measured in a liquid scintillation analyzer (Tricarb 2000 CA, Hewlett-Packard, Palo Alto, CA). Overactivity was measured in a liquid scintillation analyzer.

Measurement [14C]-CO₂ Production by [14C]-Man-Fed Root Tips

One-hundred excised maize root tips were incubated as described in the first paragraph, for 2 d, in a 20-mL syringe containing 10 mL of medium A supplemented with antibiotic-antimycotics, antifoam A (ref A-5758, Sigma), and 10 mM [U-14C]Man (3.7 MBq/mmol). The syringe was connected on-line with three assay tubes, each containing 2.5 mL of 2% (w/v) KOH. With this system, more than 98% of the CO₂ produced by the root tips was trapped in the KOH solution. The three assay tubes were changed every 4 to 8 h. The radioactivity in the KOH solution, due to [14C]CO₂ production, was measured in a liquid scintillation analyzer. The amounts of Man metabolized through the respiration were calculated from the [14C]CO₂ production and the specific activity of [14C]Man.

Perfusion of Maize Root Tips

For in vivo NMR, 3,000 root tips were placed between two filters into a 25-mm tightly closed NMR tube, part of a homebuilt perfusion system. The latter, evolved from the experimental device described previously (Roby et al., 1987), allows circulation of a nutritive medium controlled in solute composition, temperature, and pH, through the living sample. For the present study, temperature and pH were regulated at 21°C and 6.5, respectively. The partial oxygen pressure (50%) in the external medium was established by bubbling a mixture of oxygen and nitrogen into the medium reservoir. These environmental parameters were measured continuously on-line using a thermocouple, pH, and oxygen electrodes inserted in a by-pass device. By measuring the partial oxygen pressure in the circulating medium before and after the sample sequentially with the same electrode, respiration rates were determined during the NMR experiments.

Preparation of Acid Extracts

For in vitro NMR, 2,000 tips were incubated for 48 h in flasks containing the required incubation medium flushed with a 50% O₂ + 50% N₂ gas mixture. The medium was renewed every 12 h. At the end of this treatment, it took 10 s to gather the root tips on a sieve, rinse them with distilled water, and throw them into a mortar filled with and surrounded by liquid nitrogen. The frozen tissues were ground with mortar and pestle into a fine powder, adding slowly 3.9 m perchloric acid (PCA), which was thoroughly mixed in the powder. The sample was adjusted for a final PCA concentration of 1 m.

The powdered mixture was transferred into a 50-mL centrifuge tube cooled in liquid nitrogen and later thawed and stirred at ice temperature. After addition of another 10-mL/5-g fresh matter of 1.1 m PCA, the mixture was centrifuged at 25,000 g for 15 min. This operation was repeated twice, the pellets being homogenized with the same volume of PCA for the first time and water for the second. The three supernatants were mixed with 5 mL of a 2 m potassium bicarbonate solution cooled on ice. The pH of the resulting solution was adjusted to 5.0 by dropwise addition of 2 m KHCO₃. The KClO₄ precipitate was eliminated by centrifugation at 17,000 g for 20 min. The supernatant was lyophilized and the resulting powder dissolved into 10 to 15 mL of deionized water. The resulting suspension was centrifuged as described above and the last supernatant lyophilized.

The final dry extract was dissolved into 1.8 mL water + 0.2 mL D₂O with a concentrated Tris-base buffer 35 mm final at pH 7.5. The solution was frozen in liquid nitrogen, thawed, and centrifuged at 25,000 g for 15 min. The pH of the supernatant was adjusted to 7.5 using concentrated KOH or HCl, and the volume adjusted to 3.0 mL with water containing 10% (v/v) D₂O. This sample was first used for 13C-NMR analysis. Before 31P-NMR analysis, a 500 mM CDTA (cyclohexane diamine tetraacetic acid) solution was slowly added until pH stabilized to 7.5. A freeze-thaw cycle was also performed using liquid nitrogen, and the solution was centrifuged at 25,000 g for 15 min.

Preparation of Crude Extracts

Frozen root tips were crushed at 4°C in a glass potter with grinding medium (0.3 mL for 50–100 root tips) containing 20 mM HEPES, pH 7.4, 5 mM β-mercaptoethanol, and 0.3% (w/v) insoluble polyvinyl polypyrrolidone. The brei was transferred to a 1.5-mL microcentrifuge tube and the potter was rinsed with 0.3 mL grinding medium that was then pooled with the brei. The homogenate was centrifuged at 25,000 g for 15 min. The supernatant was used for protein and enzymic activity measurement and for immunodetection experiments.

Enzyme Activity Assays

Endopeptidase activities were measured, at pH 6.1, against azocasein as described in James et al. (1993). The extinction coefficient E 1% (v/v) azocasein in 1 m NaOH = 37 L
cm\(^{-1}\) g\(^{-1}\) was used to calculate the azocaseinase activity. MPI activity was measured using a coupled enzymatic assay (Stein and Hansen, 1999). Reaction mixture (1 mL final volume) contained 50 mM Tris-HCl, pH 7.6, 5 mM MgCl\(_2\), 2 units phosphi-Glc isomerase, 1 mM NAD, 2 units NAD-dependent Glc-6-P dehydrogenase, and 50 to 150 µL of enzymic extract. The reaction was started with 2 mM Man-6-P, and it was monitored spectrophotometrically at 340 nm.

Electrophoresis and Immunological Methods

SDS-PAGE were performed with 12.5% (w/v) polyacrylamide gels by the procedure of Laemmli (1970). Proteins were transferred from SDS-gel to a nitrocellulose membrane (BA 85, Schleicher and Schull) for 1 h at 3 mA/cm\(^2\). The residual blot was soaked in 0.5 M Tris-HCl, pH 7.6, 50 mM MgCl\(_2\), 2 units phosphi-Glc isomerase, 1 mM NAD, 2 units NAD-dependent Glc-6-P dehydrogenase, and 50 to 150 µL of enzymic extract. The reaction was started with 2 mM Man-6-P, and it was monitored spectrophotometrically at 340 nm.

Protein Determination

Proteins were quantified according to the method of Bradford (1976) using the Bio-Rad Laboratories microassay reagent. Bovine γ-globulin was used as the protein standard.

Extraction and Determination of Solubles Sugars, Total Amino Acids, and Adenine Nucleotides

Solubles sugars and free amino acids were extracted by boiling ethanol/water procedure (Stitt and Rees, 1978), and analyzed according to Moing et al. (1992) and Brouquisse et al. (1992), respectively. Adenine nucleotides were extracted by the diethyl-ether/trichloroacetic acid procedure and determined by the bioluminescence method according to Salewski et al. (1992), plus goat anti-(rabbit IgG)-alkaline phosphatase conjugate.

For immunoprecipitation experiments, 1 mL of each crude extract supernatant was desalted by centrifugation through G-25 Sephadex (Pharmacia Biotech, Piscataway, NJ) column, equilibrated with 50 mM MES-Na, pH 6.1, and 20 mM NaCl. Aliquots of 200 µL were incubated for 2 h at 25°C with increasing volume of immune or preimmune anti-RSIP serum. Immune complexes were incubated for 1 h at 6°C with a 2-fold (IgG-binding) excess of Protein A-Agarose (Bio-Rad Laboratories) and then centrifuged for 10 min at 10,000g. The azocaseinase activity was then measured in each supernatant fraction.

NMR Spectroscopy

The NMR experiments were done with an AMX400 WB spectrometer (Bruker SA, Wissembourg, France). Phosphorous and carbon spectra were acquired at 162 and 100 MHz, respectively. A D\(_2\)O signal was used to lock and shim the static magnetic field. Acid extracts were analyzed at 21°C using a 10-mm broadband probe, whereas \(^{31}\)P and \(^{13}\)C spectra of living root tips were recorded alternatively using a dual electronically-switched 25-mm probe. Proton decoupling during signal acquisition was realized using a Waltz 16 sequence and a proton radiofrequency power of 0.5 W. A 0.05-W proton irradiation was applied during the relaxation delay to maintain nuclear overhauser enhancement for routine spectra but not for relaxed spectra. These conditions applied to both nuclei and were the same in vivo and in vitro.

Carbon NMR

The resonance assignments were made on the basis of their chemical shifts using published data (Johnson and Jankowski, 1972; Genix et al., 1990; Fan, 1996) and by comparison with carbon spectra of the pure compounds. Chemical shifts were referenced to tetramethylsilane using the C2 resonance of the fructosyl moiety of Suc, fixed at 104.4 ppm, as a secondary reference. With acid extracts, the molecular concentrations were determined from the resonance intensities of the relaxed spectra taken as peak areas. A 3-(trimethylsilyl) propionic acid-d4 sodium salt solution contained in a concentric capillary provided an external calibrated reference. In vivo, the measured resonance intensities of Asn were calibrated against concentration using the value determined in vitro for root tips submitted to 48-h carbon starvation.

Phosphorous NMR

The resonance assignments were made using published data (Roberts, 1984; Roby et al., 1987; Saint-Ges et al., 1991) and comparison with spectra of the pure suspected compounds. In a limited number of cases (3PGA, F6P, and P-eth, M1P, M6P, and UDP-Man) definitive identifications were obtained using the coresonance and titration techniques. Chemical shifts were referenced to 85% (v/v) H\(_3\)PO\(_4\) using internal glyceral phosphoryl choline as a secondary reference (−0.05 ppm) for acid extract spectra and a 50 mM methylidiphosphonate solution at pH 8.9 in 100 mM TRIS contained in a coaxial capillary as a secondary reference (16.38 ppm) for in vivo spectra. Quantification was done by integration of the resonances but their overlap at pH 7.5 prevented integration of isolated peaks in the following three cases. The measured Pi intensity contains the contribution of the G1P resonance, although much weaker. P-eth and F6P resonances are superimposed and the 3PGA one is certainly not unique at this chemical shift. G6P and M6P resonances could not be integrated separately when one of them was much greater than the other. In these three cases, therefore, the intensity measured at a given chemical shift was attributed to the major compound on biochemical basis. For in vivo experiments, first peak areas were measured and plotted as a function of time, second the data were calibrated using the intensities of the control spectrum of maize root tips fed on 200 mM Glc in the following way. The raw intensities of the resonances were corrected for the saturation effect arising from the fast acquisition procedure and for the nuclear Overhauser enhancement during the relaxation delay. The
corrected intensities were finally calibrated against concentrations using 1 mM for the ATP concentration value, determined biochemically for Glc-fed root tips (Saglio and Pradet, 1980; Brouquisse et al., 1991).

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