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# Glycolytic Flux and Hexokinase Activities in Anoxic Maize Root Tips Acclimated by Hypoxic Pretreatment

**Jean M. Bouny and Pierre H. Saglio***

Station de Physiologie Végétale, Institut National de la Recherche Agronomique, Centre de Recherches de Bordeaux, B.P. 81, 33883 Villenave d'Ornon Cedex, France

**Several enzyme activities were measured in extracts from acclimated and nonacclimated maize (*Zea mays*) root tips at pH 6.5 and 7.5, corresponding to cytoplasmic pH in anaerobiosis or aerobiosis, respectively, to determine what causes the decline of the glycolytic flux observed in anoxia in nonacclimated tips. We found that phosphorylation of hexoses by kinases was a major limiting step of glycolysis in anoxia. When fructose was substituted for glucose, glycolysis was slightly enhanced and survival improved, but neither matched that of acclimated tips. Decrease of kinase activities was not the result of proteolytic degradation but was more likely the result of inhibition by internal factors (low pH and low ATP). There was no evidence of induction during the hypoxic pretreatment of isoenzymes better adapted to the anoxic cellular environment. Maintenance of the glycolytic flux in acclimated tissues is explained by a combination of a rise in kinase activities and decreased inhibition resulting from a higher cytoplasmic pH and ATP content. The behavior of intact root tips is discussed in comparison with the behavior of excised root tips.**

A preacclimation in hypoxia allows excised maize (*Zea mays*) root tips supplemented with external Glc to survive several days in strict anoxia instead of less than 10 h in the absence of pretreatment (Saglio et al., 1988; Xia and Saglio, 1992). However, in the absence of exogenous Glc, they do not survive significantly longer than nonacclimated tips. We have shown that, below a critical threshold of glycolytic flux, survival was compromised (Xia et al., 1995). An hypoxic pretreatment induces an increase of maximal in vitro catalytic activities of many enzymes involved in sugar metabolism, a higher ATP level correlated with a higher energy charge (reviewed by Ricard et al., 1994), and a better regulation of cytoplasmic pH linked to an efficient efflux of lactic acid (Xia and Roberts, 1994; Xia et al., 1995; Xia and Saglio, 1992). However, the role of these modifications in the subsequent survival of tissues in anoxia is not obvious. For example, we have shown that in HPT root tips the level of ATP is not critical for survival or for cytosolic pH regulation in anoxia (Xia et al., 1995), and the increase in glycolytic enzyme activities is not correlated with a higher glycolytic rate during the 90 min after the transfer of tissues from air to strictly anoxic conditions. During this period, the glycolytic rate (measured as the sum of ethanol plus lactate) remains strictly identical in HPT and NHPT root tips. It is only after 60 to 90 min that the glycolytic flux declines and then almost stops in NHPT root tips even in the presence of added Glc, whereas it remains sustained in HPT tips (Xia and Saglio, 1992). Such a decline of ethanol production during the hours or days after the transfer to anoxia appears to be common to many plants (Smith and ap Rees, 1979; Raymond et al., 1985; Kimmerer and Mac Donald, 1987). Exceptions are rice seeds and seedlings, which are resistant to anoxia and in which the rate of ethanol production increases during at least the first 2 d in the absence of $O_2$ (Raymond et al., 1985).

How glycolytic flux is modified in HPT and NHPT root tips and what is responsible for its decline in NHPT tips early in anoxia (Hole et al., 1992; Xia and Saglio, 1992) remain to be elucidated and are the subject of this paper. We have studied whether HKs (GK and FK) control sugar utilization under anoxia. HK phosphorylating activities were determined in vivo, as were their stability during prolonged incubation in anoxia. Excised root tips were compared with intact root tips of young seedlings. The results obtained permit a better understanding of the role of increased enzyme activities observed after hypoxic acclimation in relation to subsequent survival in anoxic conditions.

## MATERIALS AND METHODS

### Plant Material and Pretreatments

Maize (*Zea mays* L., Dea, Pioneer, Hi-Bred [Pioneer, France-Maïs, Toulouse, France]) seeds were germinated in the dark at 25°C on wet filter paper. Three days after imbibition, germinated seedlings (with seminal roots 6–8 cm long) were placed on rafts floating on 7 L of nutrient solution (Saglio and Pradet, 1980) in a gas-tight plastic tank equipped with inlet and outlet fittings, which allow the solution to be bubbled through sintered glass blocks with appropriate gas mixtures (50% and 3%, $O_2$ in $N_2$, for NHPT and HPT, respectively). The pretreatments usually lasted

* Corresponding author; e-mail saglio@bordeaux.inra.fr; fax 56–84–32–45.

Abbreviations: ADH, alcohol dehydrogenase; dGlc, 2-deoxyGlc; dGlc-P, 2-deoxyglucose phosphate; Eno, enolase; FK, fructokinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GK, glucokinase; HK, hexokinase; HPT, hypoxically pretreated; LDH, lactate dehydrogenase; NHPT, not hypoxically pretreated; PDC, pyruvate decarboxylase; PFK, ATP:Fru-6-P phosphotransferase; PFP, PPi:Fru-6-P phosphotransferase; PGI, phosphoglucose isomerase; pHc, cytosolic pH; PK, pyruvate kinase; TPI, triose phosphate isomerase.

overnight (16 h), and then 5-mm excised primary root tips (3.2 mg fresh weight per tip) were used in most experiments, unless otherwise stated.

### Anaerobic Treatments

Root apices were placed in disposable syringes (10 mL) containing the above nutrient medium buffered with 10 mM Mops (pH 6.2) and supplemented or not with 100 mM Glc, Fru, or Suc. The syringes with rubber puncture caps were fitted with 12 × 0.45-mm needles onto rubber vacuum tubing flushed with $N_2$ containing less than 4 $\mu$L $L^{-1}$ $O_2$.

### Survival and Adenine Nucleotide Determination

Viability was assessed as the ability of anoxic root tips to recover their ATP content after 30 min of return to air, as described in Xia and Saglio (1992). The root tips were considered dead when their residual ATP content represented 10% or less of the value found before the anoxic treatment; such low values corresponded to a lost of turgidity.

ATP represents 90% of the adenine nucleotide pool in normal, respiring tissues, and its level remains very stable and close to 1 mM in most cells. Because of its rapid turnover (a few seconds) and direct link to the functioning of cell metabolism, ATP has been used successfully to measure growth and viability of microorganisms and animal tissues (for review, see Pradet and Raymond, 1983) and has proven to be quantitatively related to the number of viable plant cells (P.H. Saglio, unpublished data). It takes less than 10 min for ATP to return its normoxic level when undamaged anoxic root tips are transferred in air. This technique has already been used to study the viability of maize roots (Saglio et al., 1988; Xia and Saglio, 1992) and was preferred for excised tissues, for which growth measurements cannot be easily made.

### Determination of Lactic Acid, Ethanol, and Sugars

For lactic acid and sugar determinations, 25 root tips were incubated in 1 mL of medium flushed continuously with humidified $N_2$ gas as for anoxic treatments. After various incubation times, the medium was collected and the root tips rapidly rinsed. All samples were frozen before assay.

For ethanol determinations, 25 root tips were incubated in 10 mL of medium containing 100 mM Glc or Fru and 100 $\mu$g of ampicillin. Anoxia was obtained by bubbling the medium for 5 min with $N_2$ before starting the experiment in a confined atmosphere. Aliquots of the medium were collected at different times and immediately frozen until assayed.

Frozen tissues were ground in 5-mL conical glass homogenizers (Prieux, Joinville-le-Pont, France) with 0.8 mL of 10% perchloric acid, left for 30 min at 4°C before neutralization with 0.24 mL of 5 M KOH in 1 M triethanolamine, kept on ice for another 15 min, and finally centrifuged for 5 min at 6000*g* in an Eppendorf microfuge. Lactic acid, ethanol, and soluble sugars were determined enzymatically on incubation medium or tissue extracts according to Saglio et al. (1980).

### Enzyme Activities

All extractions were done on ice. After the different treatments, 50 root tips were ground in a 5-mL conical glass homogenizer with 0.4 mL of ice-cold extraction buffer consisting of 50 mM Tris/HCl (pH 7.5), 10 mM sodium borate, 5 mM DTT, 15% glycerol, and 5 mg $mL^{-1}$ BSA. The slurry was centrifuged for 5 min at maximum speed in an Eppendorf microfuge, and the supernatant was immediately desalted by a 2-min centrifugation at 1400*g* on a superfine Sephadex G-25 column equilibrated with the extraction buffer (Helmerhost and Stokes, 1980).

Enzyme activities were assayed at 25°C in 100 mM Tricine buffer adjusted to pH 7.5 or 6.5 in a final volume of 1 mL. NAD reduction or NADH oxidation was recorded at 340 nm before and after starting the specific reaction to correct for nonspecific activity. In all cases, activity was linearly related to time and amount of extract. In addition, the assay for each enzyme was authenticated by optimizing the concentration of each component of the reaction mixture. For GK and FK, the reaction contained 2 mM $MgCl_2$, 3 mM DTT, 0.5 mM NAD, 1 mM ATP, and 20 nkat of Glc-6-P dehydrogenase; 1 mM Glc or 1 mM Fru and 80 nkat of PGI were added for the detection of GK or FK, respectively. The reaction was initiated with the addition of ATP. For PFP, the reaction contained 2 mM $MgCl_2$, 0.2 mM NADH, 5 mM Fru-6-P, 5 $\mu$M Fru-2,6-$P_2$, 1 mM PPi, 17 nkat of aldolase, 170 nkat of TPI, and 17 nkat of glycerol-3-P dehydrogenase. The reaction was initiated by the addition of Fru-6-P. For PFK, the conditions were similar to those for PFP except that Fru-2,6-bisP was omitted, and 1 mM ATP replaced PPi. For GAPDH, the reaction contained 2 mM $MgCl_2$, 2 mM EDTA, 5 mM DTT, 0.2 mM NADH, 5 mM glycerate-3-P, 1 mM ATP, 170 nkat of TPI, and 85 nkat of 3-phosphoglycerate kinase. The reaction was initiated by the addition of glycerate-3-P. For Eno, the reaction contained 2 mM $MgCl_2$, 10 mM KCl, 1 mM ADP, 0.2 mM NADH, 1 mM glycerate-2-P, 28 nkat of PK, and 85 nkat of LDH. The reaction was initiated by addition of glycerate-2-P. For PK, the reaction contained 2 mM $MgCl_2$, 40 mM KCl, 2 mM DTT, 0.2 mM NADH, 1 mM ADP, 1 mM PEP, and 85 nkat of LDH. The reaction was initiated by the addition of PEP. For PDC, the reaction contained 2 mM $MgCl_2$, 1 mM thiamine pyrophosphate, 10 mM DTT, 0.2 mM NADH, 25 mM oxamate, 10 mM pyruvate, and 51 nkat of ADH. The root-tip extract was incubated for 1 h in the reaction medium in the absence of pyruvate for PDC activation before starting the reaction by the addition of pyruvate. For ADH, the reaction contained 0.4 mM NAD and 100 mM ethanol. The activity was measured in the ethanol-to-acetaldehyde direction and was initiated by the addition of ethanol. For LDH, the reaction contained 100 mM pyrazole, 10 mM KCN, 0.2 mM NADH, and 12 mM pyruvate. The activity was measured in the pyruvate-to-lactate direction in the presence of pyrazole to inhibit the coupled reaction of PDC and ADH, and the reaction was initiated by the addition of pyruvate.

### HK Activity Gels

Root tips were extracted as described above for measurement of enzyme activities, but BSA was not included in the extraction buffer. Kinase isoenzymes were separated by electrophoresis at 4°C on native polyacrylamide gels using the discontinuous system described by Laemmli (1970) in the absence of SDS. The pH of both stacking and separating gels was 8.8, and the concentration of acrylamide was 4.75 and 8%, respectively. Protein in the extracts was determined by the method of Bradford (1976), and 25 $\mu$g of protein extract were deposited in each lane.

Enzymes were visualized by incubating the gels at 37°C in 20 mL of the reaction medium described above for the measurement of HK and FK activities in the presence of 6.5 mM phenazine methosulphate and 0.4 mM nitroblue tetrazolium.

### Analysis of Sugar-P

Glc-6-P and Fru-6-P in perchloric extracts of 50 root tips (as described above for ethanol, lactic acid, and sugars) were assayed enzymatically in 1-mL reaction mixtures according to Burrell et al. (1994).

### Phosphorylation of dGlc

The rate of in vivo phosphorylation was assessed using a labeled, nonmetabolizable analog of Glc, dGlc (2-deoxy-D-[U-$^{14}$C]Glc, 11.8 GBq mmol$^{-1}$; Dupont/NEN). Lots of 40 HPT or NHPT root tips were added to 10-mL syringes containing 10 mL of the medium supplemented with 100 mM Glc and incubated for 5 h in anoxia as described above. They were then rapidly rinsed three times within 20 s with the nutrient medium to remove any trace of Glc and were transferred to 2 mL of nutrient medium (without Glc) flushed with air or $N_2$. After 15 min, 2 mM labeled dGlc (74 MBq mmol$^{-1}$, 148 kBq mL$^{-1}$ final concentration) was added to the anoxic medium, and only one-quarter of this radioactivity was added to the aerated medium. Similarly, freshly excised root tips were incubated in aerated solution as a control. After 30 min (time during which the absorption rate was linear), root tips were rinsed and immediately frozen. The labeled compounds were extracted as for soluble sugars and separated by TLC as described by Saglio (1985). After a 3-d exposure period on Kodak ARD x-ray safety film, two radioactive spots identified as dGlc and dGlc-P were located on the chromatogram and recovered for counting to calculate the percentage of total radioactivity absorbed corresponding to phosphorylated dGlc.

Alternatively, 400 $\mu$L of labeled extract were mixed with 200 $\mu$L of $HCO_3^-$ anionic resin (Bio-Rad AG 1X8, 200–400 mesh). The radioactivity was measured before and after the resin treatment. The difference was attributed to the fixation of dGlc-P by the resin. Similar treatments were done with pure, labeled dGlc to assess the dilution due to the added resin. Controls were done to verify that the fixation of dGlc-P was total by checking the stability of the radioactivity in the solution after addition of extra resin.

Both methods gave similar conclusions, but the percent phosphorylation was regularly 10 to 12% higher with the TLC method, probably due to an underestimation of the total radioactivity by this method. The data presented are those obtained by the resin method.

## RESULTS

### Activities of Glycolytic and Fermentative Enzymes

Hypoxic pretreatment of intact seedlings induced an increase in the in vitro activities of most enzymes extracted from root tips (Table I). Only PFP was not modified by the pretreatment. After excised root tips had been exposed to 6 h of anoxia, most of the activities were unchanged or only slightly modified. The activity of most enzymes was lower at pH 6.5, which corresponds to the value found in anoxic maize root tips, than at pH 7.5, corresponding to the pH of well-aerated tissues (Saint-Ges et al., 1991; Xia and Roberts, 1994; Xia et al., 1995). Only PDC was more active at low pH, whereas PFK, PFP, and PK were not affected by pH.

Of the enzymes studied, GK had the lowest activity and was strongly inhibited at pH 6.5. To ascertain that the low values found in six independent repetitions were not the result of losses during extractions and assays, recovery experiments were carried out. In four independent trials in which amounts of pure commercial enzyme comparable to that found in root tips were added to the frozen tissues before grinding, the recoveries were in the range of 75 to 84%. GK activity increased 3-fold after the hypoxic pretreatment. FK activity was also sensitive to low pH and was slightly enhanced (1.2-fold) after the pretreatment, but its basal activity in NHPT root tips was 7 times higher than GK activity. HKs (including GK and FK) are probably regulated in a very effective manner in anoxic tissues (Renz and Stitt, 1993), so we focused our investigation on these enzymes. Both remained stable during the 6 h of anoxic incubation.

### Native Gel Electrophoresis

HPT was associated with a pronounced increase in activity bands for GK but not for FK (Fig. 1). After 6 h of anoxia, there was no difference either in pattern or in intensity, which is in agreement with the in vitro measurements done on the extracts (Table I). In the presence of 1 mM Glc or 1 mM Fru, two and three bands were detected, respectively. The faster of the three bands in Fru seemed to be the same as the faster one obtained in Glc. In conclusion, separation on native gels allowed the detection of two GKs (using preferentially Glc) and two FKs (using exclusively Fru). No new isoforms of these enzymes were observed after hypoxia or anoxia; however, the resolution of such gels might have been insufficient to definitively exclude this possibility.

### Changes in Soluble Sugar and Sugar-P Content

Soluble sugars were assayed in the tissues during the course of anoxic incubation in the absence or in the presence of 100 mM Glc (Fig. 2). At the time of excision, Glc was the predominant sugar in NHPT root tips, followed by Suc and only trace amounts of Fru. In HPT root tips, the to-

**Table I.** *In vitro enzyme activities at two different pH values measured in extracts of NHPT and HPT maize root tips before and after anoxia*

Data are means ± SD for $n > 2$ or ± the range for $n = 2$; $n$ is the number of independent repetitions indicated in parentheses.

| Enzyme | NHPT Root Tips, Time in Anoxia | | | | HPT Root Tips, Time in Anoxia | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 h | | 6 h | | 0 h | | 6 h | |
| | pH 6.5 | pH 7.5 | pH 6.5 | pH 7.5 | pH 6.5 | pH 7.5 | pH 6.5 | pH 7.5 |
| | *nmol tip$^{-1}$/h$^{-1}$* | | | | | | | |
| GK | 16 ± 2(6) | 54 ± 8(6) | 10 ± 1(2) | 38 ± 2(2) | 101 ± 34(6) | 179 ± 37(6) | 100 ± 39(2) | 207 ± 31(2) |
| FK | 174 ± 27(6) | 399 ± 138(6) | 198 ± 25(2) | 321 ± 75(2) | 228 ± 24(6) | 491 ± 113(6) | 242 ± 63(2) | 414 ± 136(2) |
| PFK | 148 ± 38(3) | 189 ± 60(3) | 141 ± 37(2) | 151 ± 67(2) | 266 ± 63(3) | 280 ± 75(3) | 161 ± 74(2) | 106 ± 53(2) |
| PFP | 2,041 ± 245(2) | 2,229 ± 577(2) | 2,519 ± 111(2) | 2,596 ± 146(2) | 2,168 ± 145(2) | 2,625 ± 225(2) | 2,649 ± 365(2) | 2,756 ± 428(2) |
| GAPDH | 3,910 ± 210(2) | 7,480 ± 60(2) | 5,920 | 10,070 | 9,560 ± 3,420(2) | 14,285 ± 1,645(2) | 10,470 | 17,240 |
| Eno | 2,546 ± 204(2) | 4,446 ± 608(2) | 2,420 ± 252(2) | 3,572 ± 444(2) | 3,336 ± 752(2) | 5,712 ± 715(2) | 3,420 ± 520(2) | 5,101 ± 175(2) |
| PK | 1,441 ± 190(4) | 1,349 ± 17(4) | 1,997 ± 236(4) | 1,952 ± 104(4) | 2,149 ± 41(4) | 2,251 ± 69(4) | 2,640 ± 314(4) | 2,454 ± 150(4) |
| PDC | 195 ± 6(2) | 41 ± 25(2) | 133 ± 6(2) | 51 | 1,053 ± 60(2) | 470 ± 65(2) | 989 ± 54(2) | 567 ± 9(2) |
| ADH | 163 ± 44(3) | 417 ± 122(3) | 135 ± 33(2) | 306 ± 51(2) | 663 ± 122(3) | 1,329 ± 362(3) | 174 ± 14(2) | 949 ± 140(2) |
| LDH | 169 ± 45(2) | 259 ± 7(2) | 59 ± 20(2) | 109 ± 18(2) | 282 ± 74(2) | 442 ± 65(2) | 257 ± 62(2) | 390 ± 10(2) |

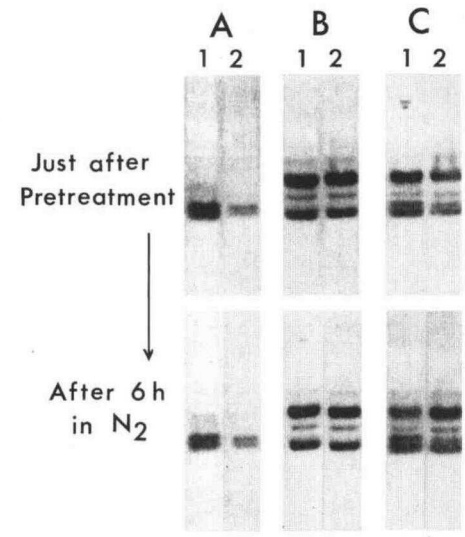


**Figure 1.** Effect of hypoxic pretreatment on HK in maize root tips. Enzymes were separated by native PAGE and visualized in the presence of 1 mM Glc (A), 1 mM Fru (B), or 1 mM Glc plus Fru (C). Lanes corresponded to 25 $\mu$g of protein from HPT (acclimated) root tips (1) or NHPT (nonacclimated) root tips (2).

tal amount of sugars was almost identical, but the proportion between Glc and Suc was reversed, with Suc being dominant. It is remarkable that the pattern of sugar utilization in anoxia was almost independent of the presence of external Glc. The only exception was in the NHPT root tips supplemented with Glc, in which the Glc level was nearly constant instead of decreasing slightly as in other treatments. In every case, there was a sharp decline in Suc, which was exhausted after 2 or 4 h in NHPT or HPT root tips, respectively.

Glc-6-P and Fru-6-P were assayed in NHPT (Table II) or HPT (Table III) excised or intact root tips during incubation in anoxia in the presence or in the absence of supplemented sugars. In recovery experiments carried out in parallel, known amounts of sugar-P were added to the frozen samples before grinding and extraction. The amounts added were comparable to those found in root tips. Four independent assays gave recoveries ranging from 73 to 92%. The amount of the different sugar-Ps was very similar in NHPT or HPT root tips at the end of the pretreatment (time 0), with Glc-6-P being largely dominant. Fifty minutes after the transfer to anoxia, the pool of Glc-6-P had dropped dramatically in excised NHPT tips despite the presence of 100 mM Glc in the incubation medium. In contrast, after a small drop, the level of Glc-6-P remained high and stable in intact and excised HPT tips and still represented 60% of the initial pool after 4 h in anoxia. Fru-6-P decreased also but more slowly than Glc-6-P. In excised NHPT root tips supplemented with Fru or Suc, the amounts of Glc-6-P and Fru-6-P still present after 240 min in anoxia were significantly higher than in excised NHPT root tips supplemented with Glc and were close to the amount found after 50 min in the latter.

The phosphorylation activity of HK, assayed in vivo using dGlc, a nonmetabolizable but phosphorylatable an-

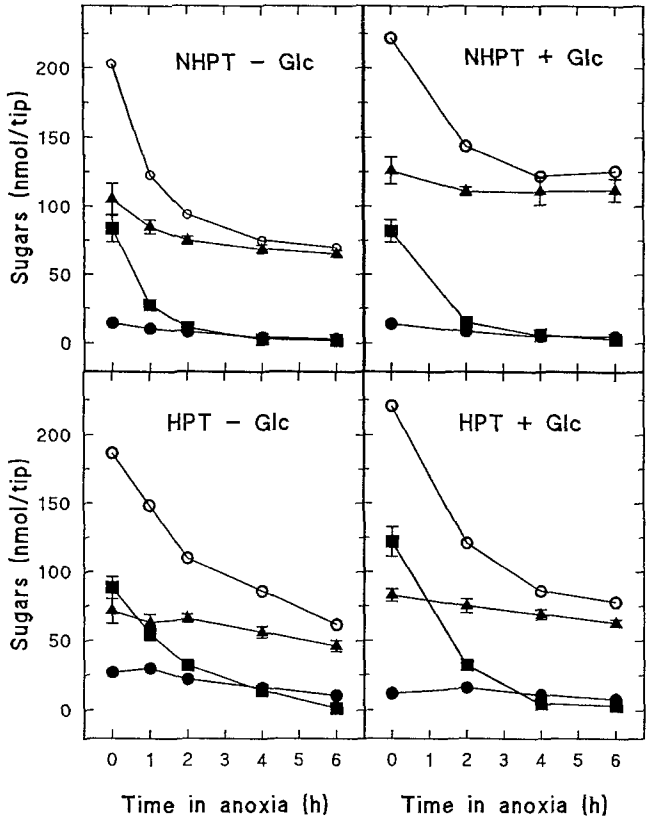


**Figure 2.** Soluble sugar content of excised maize root tips during incubation in anoxia. NHPT and HPT root tips were incubated in the presence or in the absence of 100 mM Glc. Total sugars, ○; Glc, ▲; Fru, ●; and Suc, ■. Data are given in nmol of equivalent Glc per tip. Each point represents the mean ± SD of three independent determinations.

alog of Glc, is presented in Figure 3. During the 30-min incorporation time, the penetration of dGlc was linear in both air (Xia and Saglio, 1988) and anoxia (Fig. 3, inset). The percentage of dGlc phosphorylated in 30 min, which was close to 50% and comparable in HPT and NHPT root tips in air, fell to 6% after 6 h of anoxia in NHPT root tips, whereas it remained almost unchanged in HPT tips. Note that the phosphorylating potential returned to its initial value when tissues were restored to air after 6 h in anoxia.

**Table II.** *Sugar-P content of excised NHPT maize root tips during incubation in anoxia*

Sugar-P values were determined enzymatically as described in "Materials and Methods." Zero time in $N_2$ corresponds to the end of the pretreatment period of the intact seedlings. Each point corresponds to the mean ± SD of three independent replicates.

| Treatment | Time in $N_2$ | Glc-6-P | Fru-6-P |
|---|---|---|---|
| Excised in | *min* | | |
| | 0 | 5450 ± 260 | 829 ± 110 |
| 100 mM Glc | 50 | 955 ± 87 | 178 ± 50 |
| | 240 | 182 ± 70 | 71 ± 30 |
| 100 mM Fru | 240 | 674 ± 10 | 327 ± 70 |
| 100 mM Suc | 240 | 424 ± 230 | 232 ± 130 |
| Intact | 240 | 812 ± 270 | 240 ± 160 |

### Effect of External Sugars on Glycolysis and Survival in Anoxia

During the 1st h of incubation in $N_2$, the rate of ethanol production was close to 100 nmol $h^{-1}$ per tip in both kinds of root tips regardless of the sugar added (Fig 4). After 90 min, the rate slowed down in NHPT root tips to values corresponding to only 24 or 38% of the initial rate on Glc or Fru, respectively. In HPT root tips, the rate remained unchanged on Glc throughout the experiment. After 90 min on Fru, the rate of ethanol production declined slightly and then stabilized at 68% of the rate obtained on Glc. Lactic acid accumulated to similar amounts in the presence of Fru and Glc in NHPT tissues. In HPT root tips, the total amount produced was higher but more was transported to the medium, resulting in a lower accumulation in the tissues (Fig. 4, inset).

Survival in anoxia of excised root tips, based on their ATP content, indicated that HPT root tips survived for up to 3 d on Glc as well as on Fru (Fig. 5). As expected, NHPT tips in the presence of Glc did not survive more than 12 h. The substitution of Glc by Fru significantly improved the survival of NHPT root tips. However, this improvement remained limited to about 10 h and never matched the resistance of HPT tissues.

## DISCUSSION

The enzymes studied can be classed in two groups on the basis of their activity: one group with activities higher than 1000 nmol $h^{-1}$ $tip^{-1}$ (PFP, GAPDH, Eno, and PK), the other with lower activities (GK, FK, PFK, PDC, ADH, and LDH). Given that the maximum glycolytic flux (measured as the rate of ethanol plus lactate production) during the 1st h of anoxia is close to 100 nmol $h^{-1}$ $tip^{-1}$ (or 50 nmol $h^{-1}$ $tip^{-1}$ of Glc consumed), we reasoned that enzymes having the lower activities were more likely to be involved in the limitation of glycolytic flux of NHPT root tips in anoxia than those having the higher activities. Among these enzymes, HKs (GK or FK) appeared to be particularly interesting because they were not only strongly inhibited at the pH of the anoxic cytoplasm but are known to be efficiently regulated by changes of ATP/ADP ratios (Renz and Stitt, 1993).

In NHPT root tips, the pHc drops from 7.5 to 6.4 in less than 2 h after transfer in anoxia (Xia et al., 1995). Assuming a 3-μL volume for a 5-mm root tip with an average cytoplasmic volume of 50%, ATP drops during the same time

**Table III.** *Sugar-P content of excised HPT maize root tips during incubation in anoxia*

Conditions were as described in Table II. Each point corresponds to the mean ± SD of three independent replicates.

| Treatment | Time in $N_2$ | Glc-6-P | Fru-6-P |
|---|---|---|---|
| | *min* | | |
| | 0 | 5040 ± 270 | 960 ± 300 |
| Excised in 100 mM Glc | 50 | 3081 ± 100 | 867 ± 90 |
| | 240 | 2837 ± 100 | 764 ± 120 |
| Intact | 240 | 3451 ± 126 | 1207 ± 124 |

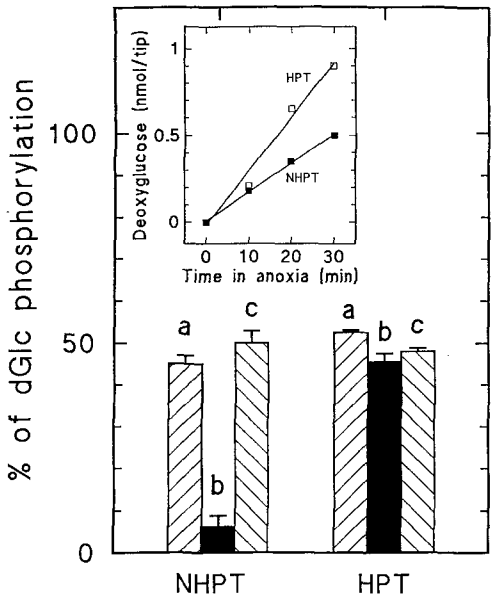


**Figure 3.** Rate of labeled dGlc phosphorylation by NHPT and HPT maize root tips expressed as percent of the dGlc absorbed (inset). Treatments shown are control in air just after pretreatment (a), after 6 h in anoxia (b), and returned to air after 6 h of anoxia (c).

from 600 to 180 $\mu$M or less, whereas ADP increases slightly from 180 to 220 $\mu$M, and AMP rises from trace amounts up to 300 $\mu$M (Saglio et al., 1988). The concentration of ATP in anoxia is thus in the range of the published $K_m$ values of maize GK (66 and 182 $\mu$M for high- and low-affinity HK, respectively; Doehlert, 1989) and is far below the $K_m$ of FK (700 $\mu$M). Thus, the low pHc and the low ATP/ADP ratios of anoxic tissues would be expected to strongly inhibit HKs (Renz and Stitt, 1993). Indeed, the data presented here (Fig. 4) show that NHPT root tips were unable to maintain a high glycolytic flux for more than 1 h in anoxia even in the presence of 100 mM Glc or Fru. This limitation cannot be explained by decreased sugar penetration in anoxia. According to Xia and Saglio (1990), sugar influx in the absence of $O_2$ is proportional to the sugar concentration and should allow the penetration of 100 and 35 nmol $tip^{-1}$ $h^{-1}$ for 100 mM Glc or Fru, respectively. A simple calculation using the penetration of labeled dGlc presented in this paper gives similar values (50 nmol $tip^{-1}$ $h^{-1}$ for NHPT root tips). Such values would provide for 100 and 70% of the maximum glycolytic rate (50 nmol $tip^{-1}$ $h^{-1}$), respectively, far above the 24 or 38% of remaining glycolytic activity actually observed after 2 h of anoxia. Neither could the arrest of glycolysis be due to proteolytic degradation of HKs during incubation in $N_2$ because phosphorylating activity was restored almost immediately to its initial value when tissues were returned to air after 6 h of anoxia, as shown by in vitro activities, native gels, and phosphorylation of dGlc (Fig. 3). The decline of glycolysis must therefore result essentially from in vivo HK inhibition as attested by the very low potential of in vivo dGlc phosphorylation (Fig. 3) and by the rapid drop in Glc-6-P even in the presence of high concentrations of Glc or Fru (Table II). The slight beneficial effect of Fru on the glycolytic rate of NHPT root tips (38 versus 24% for Glc) can be attributed to the higher initial activity of FK, which cannot, however, fully compensate for the strong limitation imposed by the low level of ATP on enzyme with a relatively low affinity for ATP (Doehlert, 1989).

HPT increased the activity of GK and to a lesser extent that of FK in root tips, as shown both by measurements of in vitro activities (Table I) and native gel electrophoresis (Fig. 1). The cytosolic environment in HPT cells is less inhibitory, with pHc regulated around 7.0 and even increasing (Xia et al., 1995) and ATP levels significantly higher than in NHPT tissues (Saglio et al., 1988). The GK activity was therefore high enough, as attested by the rapid phosphorylation of dGlc and the maintenance of an adequate pool of Glc-6-P, to sustain a high glycolytic rate. In contrast to other enzymes, such as ADH, LDH, and GAPDH (Hoffman et al., 1986; Rivoal et al., 1989), there was no evidence for the induction of isoenzymes not normally expressed in aerobic conditions, despite a lower sensitivity of HK to low pH in HPT root tips. The profile obtained was in good agreement with the separation reported by Doehlert (1989) of four forms of HK from developing maize kernels: two GKs using preferentially d-Glc and to some extend Fru, at least for one of them, and two FKs using exclusively Fru. In contrast to NHPT root tips, the relatively low rate of Fru uptake can account for its slightly lower efficiency in sustaining glycolysis in anoxic HPT root tips.

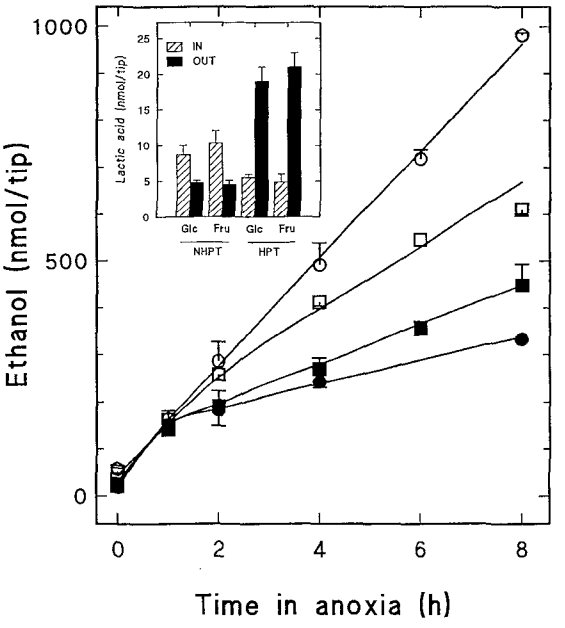


**Figure 4.** Lactic acid production and time course of ethanol production by excised maize root tips. Data are shown for NHPT tips supplemented with 100 mM Glc (●) or Fru (■) and HPT tips supplemented with 100 mM Glc (○) or Fru (□). Inset, Lactic acid was measured in the tissues (IN) or in the medium (OUT) after 90 min of anoxia. Data are the means of two independent determinations. Bars represent the ranges.

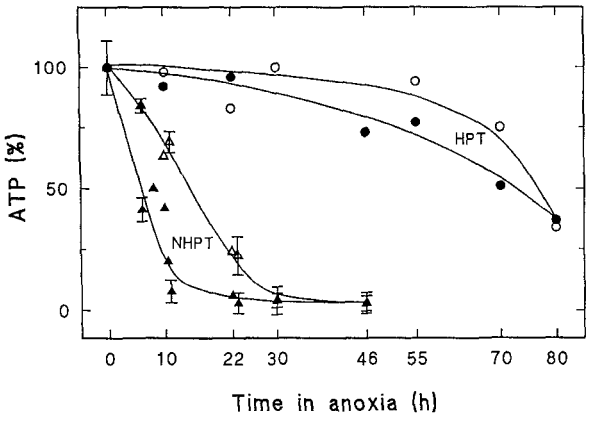


**Figure 5.** Survival in anoxia of excised maize root tips. Data are shown for NHPT tips supplemented with 100 mM Glc (▲) or Fru (△) and HPT tips supplemented with 100 mM Glc (●) or Fru (○). Points are the means of one or two independent determinations. Bars represent the ranges.

However, it remains unclear why the drop in ATP and cytoplasmic pH in anoxic NHPT root tips occurs before any significant differences in glycolytic flux are evident (Xia et al., 1995). It should be noted that the decline in glycolytic flux in NHPT root tips and to some extent in HPT tips supplemented with Fru corresponds precisely with the depletion of Suc in the tissues. On the other hand, Suc was preferentially used over Glc and Fru even by root tips supplemented with these two hexoses (Fig. 2). These findings suggest that after transfer to anoxia and until depletion of the Suc pool, Suc is the main substrate of glycolysis, allowing the bypass of the HK step by the production of Glc-1-P via the Suc synthase pathway. This would explain why the glycolytic flux remained high in NHPT root tips during the first 90 min of anoxia despite the inhibition of HKs. Root tips supplemented with external Suc behaved like tissues supplemented with Glc or Fru because Suc is poorly transported as such into maize cells and must first be hydrolyzed by cell-wall invertases (Lin et al., 1984; Xia and Saglio, 1988). It is interesting to note that with regard to Glc-6-P, that intact root tips of 3- to 4-d-old seedlings behaved like excised tips. Suc was the only form of transported sugar, so these results imply that most of the Suc could not enter the cells of intact NHPT root tips as such. Otherwise it would have been metabolized via the Suc synthase pathway to feed glycolysis, maintaining high Glc-6-P levels. These results are in good agreement with a dominant apoplastic route for sugar unloading in the primary maize root tip as predicted by the theoretical calculation of Bret-Harte and Silk (1994).

The effect of hexoses on survival in anoxia paralleled the effect on glycolysis. The slight improvement brought about by Fru on NHPT survival probably corresponded to its ability to improve glycolysis slightly. However, a glycolytic rate accounting for only 40% of the flux to ethanol in acclimated controls is below the threshold of 50% reported by Xia et al. (1995) to be required for survival and cytoplasmic pH regulation during anoxia. In addition, cell death is probably hastened in NHPT root tips because they accumulate more lactic acid (Fig. 4). By contrast, the sustained glycolytic flux of HPT root tips supplemented with Glc or Fru and their ability to effectively excrete lactic acid to the medium (limiting its accumulation in the cytosol) may be sufficient to explain survival up to 3 d in anoxia.

## CONCLUSION

The research presented here shows that HK and FK are markedly inhibited in NHPT maize root tips. This inhibition accounts for most of the decline of glycolytic flux observed in anoxic NHPT root tips. Such a high control of glycolysis by the first irreversible step of the pathway, namely the phosphorylation of hexoses, is not contradictory with the general picture emerging from the quantitative studies of metabolic control analysis developed by Kacser and Burns (1973) and Henrich and Rapoport (1974), which shows that control is usually highly shared among the glycolytic enzymes. This sharing depends very much on environmental conditions and tissues. Control coefficients of HKs as high as 0.70 to 0.90 are reported by Rapoport et al. (1974) in human erythrocytes, which are nonrespiring cells. HKs are also reported to control glycolysis in stressed animal cells (German, 1993; Kashiwaya et al., 1994; Stocchi et al., 1994), and a similar role for HK in anoxic plants tissues has been evoked by Renz et al. (1993) and Renz and Stitt (1993). It is interesting to point out that modulations of HK activities appear to function as a sugar sensor and signal transmitter able to control genome expression in higher plants (Jang and Sheen, 1994). This observation suggests that partial inhibition of HK activity in hypoxia might be the signal triggering the induction of the many enzymes observed during acclimation in hypoxia.

In maize root tips, acclimation to anoxia is the result of two main strategies. One involves a better regulation of cytosolic pH by mechanisms largely independent of ATP levels (Xia et al., 1995). The other implies the maintenance of a sustained glycolytic flux, above a critical threshold that has been estimated at 50% of the maximum rate by Xia et al. (1995), to maintain a minimum rate of supply of ATP necessary to sustain cell function. This can be achieved by increasing the cellular amount of key enzymes such as HKs in a less inhibitory cellular environment. To our knowledge, these results represent for the first time the demonstration of the significance of the hypoxic induction of enzymes involved in sugar metabolism in tolerance of plant tissues to anoxia.

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