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Enhancement of Anaerobic Respiration in Root Tips of *Zea mays* following Low-Oxygen (Hypoxic) Acclimation[1]

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ABSTRACT

Root tips (10-millimeter length) were excised from hypoxically pretreated (HPT, 4% [v/v] oxygen at 25°C for 16 hours) or nonhypoxically pretreated (NHPT, 40% [v/v] oxygen) maize (*Zea mays*) plants, and their rates of respiration were compared by respirometry under aerobic and anaerobic conditions with exogenous glucose. The respiratory quotient under aerobic conditions with 50 millimolar glucose was approximately 1.0, which is consistent with glucose or other hexose sugars being utilized as the predominant carbon source in glycolysis. Under strictly anaerobic conditions (anoxia), glycolysis was accelerated appreciably in both HPT and NHPT root tips, but the rate of anaerobic respiration quickly declined in NHPT roots. [U-^{14}C]Glucose supplied under anaerobic conditions was taken up and respired by HPT root tips up to five times more rapidly than by NHPT roots. When anaerobic ethanol production was measured with excised root tips in 50 millimolar glucose, HPT tissues consistently produced ethanol more rapidly than NHPT tissues. These data suggest that a period of low oxygen partial pressure is necessary to permit adequate acclimation of the root tip of maize to subsequent anoxia, resulting in more rapid rates of fermentation and generation of ATP.

Maize, like many other dryland species, is generally considered to be anoxia intolerant (3). When root tips of maize are anaerobically shocked, by immediate transfer from fully aerobic to anaerobic conditions, they usually die in 18 to 24 h (11, 16). However, a period of hypoxia imposed by supplying 4% (v/v) O_2 (equivalent to 4 kPa oxygen partial pressure) in the root gaseous environment results in an acclimation process that greatly improves energy metabolism (11, 20) as well as anoxia tolerance, allowing roots to survive in strictly O_2-free (anaerobic) conditions for much longer periods (11). Enhanced survival under anoxia resulting from a hypoxic pretreatment HPT[3] raises the question of the specific changes in cell physiology taking place during hypoxia. Earlier studies conducted under conditions of hypoxia or anoxia indicate a change in the pattern of proteins produced (18). About 20 proteins, including alcohol dehydrogenase, pyruvate decarboxylase, glucose-phosphate isomerase, aldolase, and sucrose synthase continue to be produced, while there is inhibition of the synthesis of all other proteins (10, 12, 13, 18, 26). All of the "anaerobic" proteins so far identified are enzymes involved in glycolysis and fermentation of carbohydrates. However, the functional significance of these changes in protein synthesis has not been directly examined in relation to acclimation to anoxia; *i.e.* whether their synthesis contributes to a greater rate of anaerobic respiration.

Survival during anoxia requires that the plant maintain some energy production without oxidative phosphorylation. There is abundant evidence that without molecular oxygen, maize roots rely on fermentation to maintain the energy status of the root tip. Root tips convert carbohydrates largely to ethanol and CO_2 (5, 6, 17, 23) and in the process regenerate NAD^+ from NADH, an essential step for maintenance of glycolysis. It has been suggested that alternative electron acceptors might recycle NAD^+ pools without ethanol production (8), but one likely candidate, nitrate, has been shown to be ineffective in this regard (20). Ethanolic fermentation seems to be essential for continued energy production during anoxia (11, 17, 20, 23), although the expected net yield of ATP, starting with glucose as a substrate, is only 2 mol/mol hexose compared with a theoretical maximum of 36 mol of ATP from aerobic respiration. An acceleration of glycolysis leading to fermentation might thus be expected to partially compensate by enhancing the rate of ATP synthesis and, possibly, contribute to prolonging the viability of cells.

Earlier studies of respiration in the tips of maize roots (4, 14) demonstrated an accelerated rate of glycolysis when transferred from air to anaerobic conditions, and other instances of this effect in a wide variety of plant tissues have been reviewed by Turner (28). However, our recent investigations of acclimation to low O_2 (11, 20) led us to question the precise conditions of pretreatment that might be involved in the maximal induction of this phenomenon in maize roots. Specifically, our objective was to examine whether a period of hypoxia might be a necessary step in acclimation, leading to induction of a more rapid rate of anaerobic respiration in maize root tips.

MATERIALS AND METHODS

Growth and Pretreatment of Seedlings

Maize, *Zea mays* L., inbred line Tx5855, was germinated for 3 d on moist paper in the dark at 25°C as previously described (11). Germinated seedlings were transferred to expanded polystyrene floats in 4-L glass jars containing 2 L 1.0 mM $CaSO_4$. The solution was sparged continuously at a rate of 200 mL min^{-1} with 40% (v/v) O_2 in N_2 to ensure that all

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[3] Abbreviations: HPT, hypoxically pretreated; NHPT, nonhypoxically pretreated; RQ, respiratory quotient.

root tissues were fully aerobic; the critical oxygen pressure for maize root tips at 25°C exceeds that in air (22). Jars had inlet and outlet tubing connections that passed through screw-top lids so that the entire seedling roots and shoot were exposed to the desired oxygen partial pressure without contamination from air. Outlet tubings from the jars terminated in a beaker of water to ensure positive pressure and to verify gas flow. These pretreatments took place at 25°C at a low PPFD (40 μmol m^{-2} s^{-1}) to minimize etiolation.

After 1 d in 40% O_2, seedlings were transferred to the dark to avoid photosynthetic O_2 production, and some were hypoxically pretreated for 18 h by lowering the O_2 concentration to 4% with the balance consisting of prepurified N_2. NHPT seedlings were maintained as before at 40% O_2 for the same duration. All gas mixtures were regulated by electronic controllers.

Measurement of Respiration

Respiration rates of HPT and NHPT root tips in 40% O_2 and in O_2-free (prepurified grade, 99.97% N_2) nitrogen were measured using a Gilson differential respirometer at 25°C. Gas exchange rates were measured on batches of 15 to 20 seminal root tips in Gilson sidearm reaction vessels (25 mL). The medium contained 50 mM glucose, 0.5 mM $CaSO_4$, and 0.5 mM KH_2PO_4 buffer at pH 5.6 in a total volume of 1 mL. Roots were excised 10 mm from the tip, blotted, immediately weighed, and transferred to the buffer solution in the reaction vessel. Before the gas exchange was measured, the reaction vessel, attached tubing, and manometer were equilibrated in a water bath for 30 min. During equilibration, vessels were purged for at least 20 min with N_2 or with 40% O_2 (balance N_2) by gassing through the sidearm port with evacuation through the manometer while it was open to the outside atmosphere. The manometer valve was then closed to begin measurements about 45 min after root excision.

Consumption of O_2 in 40% O_2 vessels was measured by trapping evolved CO_2 in 10% KOH on a filter paper wick in the center well. CO_2 evolution was calculated by subtraction of O_2 consumption from the volume changes in reaction vessels lacking KOH, in which both O_2 consumption and CO_2 evolution were occurring. In vessels with 100% N_2 atmosphere, CO_2 evolution was measured directly by volume change. All gas volumes were corrected to standard temperature and pressure and converted to moles from the relation 1 μL = 0.0446 μmol.

Measurement of Anaerobic Ethanol Production

Duplicate samples of roots were immersed in the buffered glucose solution as described and then sealed in 10-mL volume Wheaton serum vials sealed with Teflon septa. Two hypodermic needles were inserted through the septum, the first extending into the buffer solution and bubbled with 100% N_2 for 10 min to deoxygenate and the second allowed exhaust from the vial. Needles were withdrawn, and at intervals 4-μL volumes were withdrawn into a Hamilton syringe and injected into a Shimadzu Mini-2 gas chromatograph, equipped with a stainless steel column packed with Hayesep D (1.8 m length isothermal 110°C) and flame ionization detector (140°C). Quantification was by injection of volumes of ethanol solution of known concentration.

Anaerobic Respiration of Exogenously Applied [^{14}C]Glucose

Uniformly labeled [^{14}C]glucose was exogenously applied to root tips that had been prepared and placed in serum vials as described but modified to include a small well fixed to the bottom of the vial. Filter paper saturated with 10% KOH was placed in the well to capture respired $^{14}CO_2$. After the vials were gassed (described above), the needles were withdrawn and [^{14}C]glucose was injected into the buffer (final specific activity, 230 MBq $mmol^{-1}$ glucose). After 2 or 4 h, the filter paper was removed, and $^{14}CO_2$ was quantitated by liquid scintillation counting. Additionally, root tips were counted at the end of the experiment to estimate total [^{14}C]glucose uptake, being the sum of respired [^{14}C]glucose and that remaining in the root tip. Sample counts were corrected for quench and counting efficiency by counting known [^{14}C]glucose amounts on filter paper of the same size.

RESULTS

Respiration Rates of Maize Root Tips

Respiration measurements on maize root tips in the presence of 50 mM glucose (Table I) showed that aerobic evolution of CO_2 and O_2 consumption were approximately equimolar; the RQ averaged 0.92 and 1.15, respectively, for HPT and NHPT roots. This indicates that respiration was primarily of carbohydrates, presumably endogenous sugars and the exogenous glucose. The rate of O_2 consumption was similar for HPT and NHPT roots, averaging 820 μL g^{-1} fresh weight h^{-1}, which is comparable to other reports for maize root tips at or near 25°C (14, 24). The consistently larger RQ for NHPT roots thus resulted from a greater CO_2 evolution rate. In a 100% nitrogen atmosphere, CO_2 output declined with time for both treatments, but that decline was more rapid with NHPT roots (Table I).

For both treatments, the rate of glycolysis under anaerobic conditions was approximately doubled relative to the aerobic rate during the initial 30 min (Fig. 1), and presumably at least this level of enhancement would have been maintained during part of the preceding 30 min before data collection while the media were being deoxygenated. With time, stimulation of glycolysis under anoxia declined, and after 2 h values decreased below 1.0. Significantly, the collapse of anaerobic respiration was much more apparent in NHPT roots.

The cumulative anaerobic evolution of CO_2 by HPT and NHPT root tips was compared further using solutions with and without addition of glucose (Fig. 2). For NHPT roots without glucose, the initial rates of CO_2 evolution (given by the slopes of the curves) were slightly less than for HPT but declined considerably during prolonged exposure to the anaerobic medium. Evolution of CO_2 by these roots was stimulated very little by exogenous glucose. By contrast, in HPT roots, the presence of external glucose exerted a clear and continuous stimulation of CO_2 evolution. The gradual decline in anaerobic respiration in the NHPT roots suggested either that metabolism was slowing in the cells, perhaps as a conse-

Table I. *Aerobic and Anaerobic Respiration Rates by 10-mm Excised Root Tips of Maize with (HPT) and without (NHPT) an 18-h Hypoxic Pretreatment*

The buffered solution contained 50 mM glucose. Values are means (SE) for *n* respirometer flasks each containing 15 or 20 root tips.

Time Interval	Gaseous Atmosphere in Flask: 40% Oxygen — HPT O_2 uptake (n = 6)	HPT CO_2 output (n = 7)	HPT RQ[a]	NHPT O_2 uptake (n = 8)	NHPT CO_2 output (n = 8)	NHPT RQ	100% Nitrogen — HPT CO_2 output (n = 39)	NHPT CO_2 output (n = 40)
h	*μL g fresh wt^{-1} h^{-1}*							
0–0.5	618 (30)	636 (96)	1.03	564 (48)	594 (84)	1.05	411 (24)	410 (24)
0.5–1.0	768 (42)	756 (54)	0.98	774 (48)	816 (66)	1.05	290 (18)	298 (18)
1.0–1.5	786 (30)	702 (102)	0.89	870 (30)	1060 (30)	1.22	313 (18)	222 (18)
1.5–2.0	864 (54)	798 (96)	0.92	840 (48)	924 (42)	1.09	283 (12)	190 (12)
2.0–2.5	870 (30)	738 (90)	0.85	924 (30)	1150 (42)	1.24	241 (12)	140 (18)
3.0–3.5	852 (54)	786 (90)	0.92	882 (66)	1030 (78)	1.16	232 (6)	130 (18)
4.0–4.5	960 (42)	834 (30)	0.87	954 (60)	1180 (90)	1.24	208 (12)	93 (12)
5.0–5.5	864 (42)	804 (36)	0.93	840 (60)	972 (84)	1.16	197 (12)	89 (18)
Average	816	750	0.92	828	966	1.15		

[a] $RQ = \frac{\text{Volume } CO_2 \text{ output}}{\text{Volume } O_2 \text{ uptake}}$

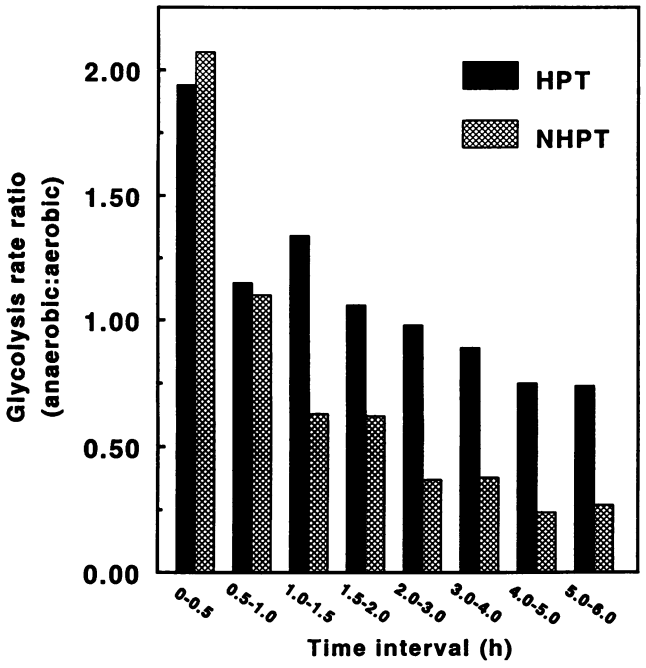

Figure 1. The ratio of the rate of glycolysis under anaerobic conditions to that under aerobic conditions. The anaerobic rate of CO_2 production was multiplied by a factor of three, to account for the one-third production of CO_2 in ethanolic fermentation relative to that in aerobic respiration, per mol of hexose respired.

quence of injury, or that transport of glucose into cells was inhibited under anaerobic conditions, so that substrate starvation was influencing CO_2 production.

Uptake and Fermentation of [^{14}C]Glucose

Rates of [U-^{14}C]glucose utilization in respiration, calculated from $^{14}CO_2$ evolution, showed an appreciable increase by HPT roots but not by NHPT roots under anoxia (Table II), compared with roots kept fully aerobic in 40% O_2. Because the rate of total CO_2 evolution in HPT or NHPT roots in 40% O_2 was 750 and 966 μL g^{-1} fresh weight h^{-1}, respectively (Table I), only 21 and 11% of this was accounted for by externally applied ^{14}C-labeled glucose (Table II). The percentage of total CO_2 released from [^{14}C]glucose was greater during anoxia in HPT roots, but the NHPT roots failed to show a comparable increase in [^{14}C]glucose respiration under anoxia.

In an experiment of 2 h duration, designed also to measure [^{14}C]glucose uptake into the roots (Table III), a twofold stimulation of [^{14}C]glucose respiration was again found when roots were made anoxic, provided that they had received an HPT. Anoxia depressed [^{14}C]glucose uptake by both sets of roots, but this effect was greater with NHPT roots. Under aerobic conditions, only a small fraction of the exogenous glucose that was taken up in 2 h was actually respired (11–13%), but with anoxia this increased substantially, particularly with HPT roots when it exceeded 50% (Table IV).

Rates of Ethanol Production

Ethanol was produced by NHPT roots during 4 h of anoxia (Table IV), but ethanol production was 60% greater in HPT roots. The corresponding release of CO_2 was also greater with HPT roots. According to the Gay-Lussac equation, there

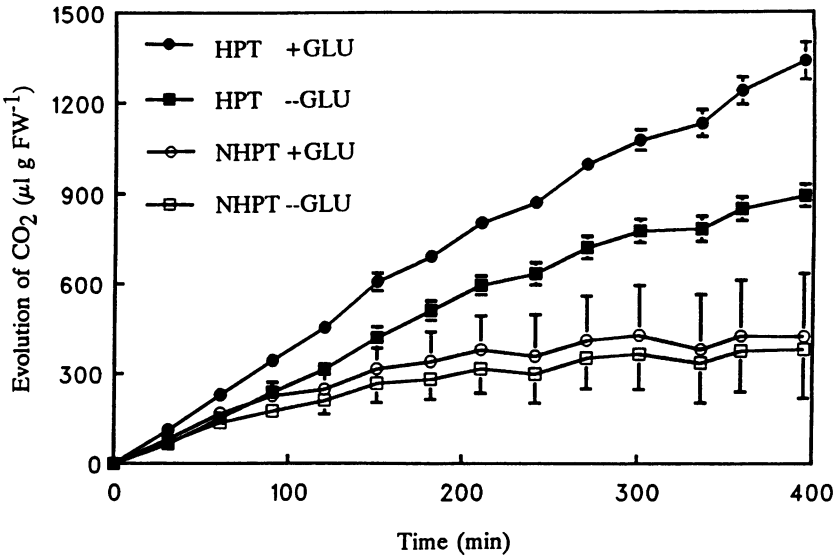

Figure 2. Anaerobic evolution of CO_2 with time by 10-mm excised root tips of *Z. mays* with and without a hypoxic pretreatment, in the presence or absence of 50 mM glucose (GLU). Each value is the mean ± SE (n = 3). FW, Fresh weight.

should be an equimolar production of CO_2 and ethanol, but for both types of root tips, CO_2 evolution slightly exceeded ethanol production. Some ethanol is likely to have been retained by the root tissue, which was not destructively sampled. However, the root tissue made up approximately 0.05 g fresh weight/mL of medium; therefore, assuming equal concentration in the tissue and surrounding medium as others have found in rice roots (1, 7), this would provide only a 5% underestimate. A further source of error may be in the production of lactate, which we did not estimate.

DISCUSSION

Faster evolution of CO_2 (Figs. 1 and 2, Table I) and greater release of ethanol (Table IV) and utilization of exogenous, ^{14}C-labeled glucose (Table II and III) all indicate the induction by hypoxia (HPT) of a more effective fermentation in anoxic root tips. No comparable response was found in anaerobically shocked roots (NHPT), although some induction of alcohol dehydrogenase (11) as well as fermentation of sugars to ethanol (Table IV) clearly took place during anoxia. However, both HPT and NHPT roots may have demonstrated a Pasteur effect during the initial stages of anoxia (Fig. 1) when the apparent rate of glycolysis was approximately doubled compared with aerobic tissues. The effect was transient however, and the most noteworthy feature was the more rapid collapse of CO_2 production in the unacclimated (NHPT) roots. Other authors have reported a Pasteur effect in maize roots (4, 14), and the possible role of phosphofructokinase and pyruvate kinase in regulation of glycolysis has been reviewed (27). Evolution of CO_2 and production of ethanol are valid measures of respiratory metabolism in anoxic maize roots. Earlier studies established that glucose breakdown occurs almost entirely by the Embden-Meyerhof-Parnas scheme, followed by fermentation to 2 mol CO_2 and 2 mol ethanol/mol glucose (5, 6). Only small amounts of lactate are formed during anaerobic incubation of maize root tips (23), and this is restricted to the initial 20 min of anaerobiosis before a lowering of intracellular pH inactivates lactate dehydrogenase and activates pyruvate decarboxylase (17).

One consequence of acclimation of maize roots to low oxygen partial pressures before excision was a greater ability to utilize an exogenous source of glucose when anaerobically incubated (Fig. 2). The unacclimated roots not only evolved CO_2 more slowly but also responded very little to exogenous glucose. The observed increase in fermentation by HPT roots was thus in part the result of greater utilization of external glucose which accounted for about one-third of the total CO_2 evolution during anoxia. Experiments with ^{14}C-labeled glucose concurred (Tables II and III); utilization of [^{14}C]glucose by unacclimated roots was strongly inhibited by anoxia com-

Table II. *Production of $^{14}CO_2$ from ^{14}C-labeled Glucose by 10-mm Root Tips of Maize*

The concentration of glucose was 50 mM during 4 h incubation under aerobic (40% O_2) or anaerobic (100% N_2) conditions. Values are means ± SE (n = 4).

Pretreatment	$^{14}CO_2$ Production		Calculated Equivalent [^{14}C]Glucose Respiration[a]		$^{14}CO_2$ as Fraction of Total CO_2 Production	
	40% oxygen	100% nitrogen	40% oxygen	100% nitrogen	40% oxygen	100% nitrogen
	μL CO_2 g fresh wt^{-1} h^{-1}		μmol glucose g^{-1} fresh wt h^{-1}		%	
HPT	155 ± 18	89 ± 7	1.15 ± 0.13	2.00 ± 0.14	20.7	31.8
NHPT	104 ± 9	17 ± 3	0.78 ± 0.07	0.39 ± 0.06	10.9	8.2

[a] Calculation based on production of 6 mol CO_2/mol glucose in aerobic respiration and 2 mol CO_2/mol glucose in fermentation.

Table III. *Uptake and Respiration of ^{14}C-labeled Glucose by 10-mm Root Tips of Maize*

The concentration of glucose was 25 mM during 2 h incubation under aerobic (40% O_2) or anaerobic (100% N_2) conditions. Values are means ± SE ($n = 4$).

Pretreatment	Calculated Equivalent [^{14}C]Glucose Respiration[a]		Total [^{14}C]Glucose Uptake		[^{14}C]Glucose Respired as Fraction of Total [^{14}C]Glucose Uptake	
	40% oxygen	100% nitrogen	40% oxygen	100% nitrogen	40% oxygen	100% nitrogen
	μmol glucose g^{-1} fresh wt h^{-1}					
HPT	0.91 ± 0.02	1.83 ± 0.16	7.03 ± 0.18	3.39 ± 0.22	12.9	54.0
NHPT	0.71 ± 0.04	0.69 ± 0.03	6.30 ± 0.41	2.11 ± 0.05	11.3	32.7

[a] Calculated from the production of $^{14}CO_2$, as in Table II.

pared with aerobic roots, in marked contrast to the faster utilization under anoxia by HPT roots.

The mechanism of uptake of glucose by plant cells is by a proton-cotransport system, driven by the activity of H^+-translocating ATPases which create a greater [H^+] external to the cell (15, 25). Improvements in energy metabolism (ATP concentration and adenylate energy charge) that are apparent during anoxia as a consequence of HPT (11, 20) are likely to influence the activity of essential ATP-utilizing enzymes. We suggested earlier (11) that improved cell viability during anoxia shown by HPT roots might be because of a sufficient supply of ATP to maintain the pumping of H^+ into the vacuole by tonoplast H^+-translocating ATPases. Cell death is closely linked to the leakage of H^+ from the vacuole to cytoplasm in energy-depleted, anoxic cells of maize root tips (16). An additional feature important to cell survival, suggested by our present results, may be the ability of HPT root cells to continue to energize plasma membrane H^+-ATPases. This would allow glucose-proton cotransport into root cells, and continued respiration of the transported glucose would sustain energy metabolism. The importance of the supply of readily transportable sugars has been known since Vartepetian and coworkers (29) showed that exogenous sugars prolonged root viability and cellular substructure in many species.

The foregoing raises the question of whether the apparent stimulation of glycolysis we observed during anoxia would take place under more natural conditions with intact roots, in the absence of an external glucose source. In intact roots, translocation of sugars in the phloem was not directly affected by anoxia (19). Rather, it was the unloading of sugars from the phloem that was arrested, leading to an inhibition of root tip development. These observations suggest that intact HPT roots might indeed continue to tolerate anoxia using endogenous sugars, provided that the energy status of the cells was adequate for phloem unloading, and for symplasmic transport of sugars into the apical meristem. Excised root tips, by contrast, quickly depleted endogenous respirable substrates and rely increasingly on exogenous sources under aerobic as well as anaerobic conditions (21, 23, 30).

Was the marked slowing of anaerobic respiration in NHPT root tips the result of a shortage of respirable substrates? The evidence we have suggests that was not the case. There was evidently adequate substrate in excised roots to maintain rapid aerobic respiration, and exogenous glucose accounted for only 11% of the respired CO_2 (Table II). Furthermore, much more [^{14}C]glucose was taken up by roots under anoxia than was respired, so that adequate substrates should have been present within cells. The conclusion seems to be that it was the utilization of both exogenous and endogenous substrates that was inhibited in NHPT roots.

In the present research, for the NHPT roots, we were careful to avoid any hypoxia that might inadvertently occur during growth. The critical oxygen pressure for maize root tips at 25°C in vigorously agitated solution is 30% (v/v) O_2 (22) (*i.e.* greater than the concentration in ambient air). Thus, cells in the center of the root are likely to become hypoxic under conditions that lead to thicker boundary layers at the root surface, that lower the oxygen partial pressure, or increase O_2 consumption rates by increasing ambient temperature (2, 9). In the experiments reported here, roots were maintained fully aerobic at 25°C by sparging the solution with 40% oxygen, thereby avoiding hypoxia.

In earlier studies, induction of anaerobic proteins in roots of maize by hypoxia or anoxia were examined, and some of these proteins were identified as enzymes of the glycolytic and fermentation pathways (see "Introduction"). However, their role in acclimation of cells to anoxia has been questioned, because of the failure of maize root tips to survive >24 h under these conditions. The present results, together with preceding papers (11, 20), provide clear evidence that induction by hypoxia of anaerobic proteins is closely associated with more vigorous glycolysis and fermentation and with improvements both in energy metabolism and in anoxia tolerance of cells in the apical zone of maize roots.

Table IV. *Evolution of CO_2 and Production of Ethanol by 10-mm Root Tips of Maize under Anoxia*

Root tips were freshly excised from plants following 18 h of hypoxic pretreatment (HPT) or from plants sparged with 40% oxygen (NHPT) and anaerobically incubated in the presence of 50 mM glucose for 4 h. Values are means ± SE ($n = 3$).

	Pretreatment	
	HPT	NHPT
	μmol g fresh wt^{-1} h^{-1}	
CO_2 evolution	10.7 ± 0.5	7.9 ± 1.0
Ethanol production	9.18 ± 1.1	5.65 ± 0.5

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