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Metabolic Acclimation to Anoxia Induced by Low (2–4 kPa Partial Pressure) Oxygen Pretreatment (Hypoxia) in Root Tips of *Zea mays*

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ABSTRACT

Young intact plants of maize (*Zea mays* L. cv INRA 508) were exposed to 2 to 4 kilopascals partial pressure oxygen (hypoxic pretreatment) for 18 hours before excision of the 5 millimeter root apex and treatment with strictly anaerobic conditions (anoxia). Hypoxic acclimation gave rise to larger amounts of ATP, to larger ATP/ADP and adenylate energy charge ratios, and to higher rates of ethanol production when excised root tips were subsequently made anaerobic, compared with root tips transferred directly from aerobic to anaerobic media. Improved energy metabolism following hypoxic pretreatment was associated with increased activity of alcohol dehydrogenase (ADH), and induction of ADH-2 isozymes. Roots of Adh1$^-$ mutant plants lacked constitutive ADH and only slowly produced ethanol when made anaerobic. Those that were hypoxically pretreated acclimated to anoxia with induction of ADH2 and a higher energy metabolism, and a rate of ethanol production comparable to that of nonmutants. All these responses were insensitive to the presence or absence of NO_3^-. Additionally, the rate of ethanol production was about 50 times greater than the rate of reduction of NO_3^- to NO_2^-. These results indicate that nitrate reductase does not compete effectively with ADH for NADH, or contribute to energy metabolism during anaerobic respiration in this tissue through nitrate reduction. Unacclimated root tips of wild type and Adh1$^-$ mutants appeared not to survive more than 8 to 9 hours in strict anoxia; when hypoxically pretreated they tolerated periods under anoxia in excess of 22 hours.

Intact plants or specific plant tissues can be classified as anoxia-tolerant or anoxia-intolerant, based on their responses to continuously anaerobic conditions (1). Of the 20 wetland species examined by Barclay and Crawford (1) using an anaerobic workbench to carefully remove all traces of oxygen from the growing environment, 13 survived in the dark for at least 7 d at 22°C, as evidenced by an ability to grow when reexposed to air. Such observations, as well as those of limited growth processes in anoxic leaves of *Schoenoplectus lacustris*, *Scirpus maritimus* and *Typha latifolia* (1), embryos and coleoptiles of rice (2, 17) and *Echinochloa crus-galli* var *oryzicola* (23, 24), suggest that a specialized metabolism must contribute to cell survival or growth in the absence of molecular O_2. The biochemical basis of anoxia-tolerant metabolism is not known, but in those anoxia-tolerant tissues in which carbohydrate metabolism has been examined in detail, including the rhizomes of *S. lacustris* and *T. latifolia* (16), the rice coleoptile (2), the mesocotyl and coleoptile of *Echinochloa crus-galli* var *oryzicola* (23, 24), cell survival is accompanied by an ethanolic fermentation and by a sustained energy metabolism.

The ability of anoxia-intolerant cells, like those of maize root tips, to survive when suddenly exposed to anaerobic conditions is highly variable, depending on ambient temperature and availability of respirable substrate (13). However, in many laboratory studies the imposition of anaerobiosis, often by vigorously deoxygenating the medium with O_2-free N_2, is complete within a few minutes, so that the possible induction of an alternative metabolic pathway would not be expressed. Estimates of the average life time of ATP in aerobic maize root tips indicate as little as 8 s (22), so that a major decline in concentration of nucleotide triphosphates is completed in a few minutes under anoxia.

In nature, O_2 concentrations in the soil water decline over hours or days (5, 29), so that cells that gradually become O_2 deficient may be able to acclimate. Few if any published studies have examined specifically the metabolic response of roots to a more gradual decrease in ambient O_2. Observations of the induction of 'anaerobic polypeptides' in maize root tips (9–11, 25) is strongly suggestive of such acclimation, but the O_2 concentrations to which cells were exposed during published experiments often were not controlled or defined closely, and were rarely, if ever, strictly anaerobic. Anaerobic polypeptides were induced by hypoxia in roots of rice ($pO_2 < 5$ kPa) and wheat ($pO_2 < 2$ kPa) continuously maintained at different constant concentrations of O_2 (3, 4). However, the significance of these proteins, which include ADH[2] and some of the glycolytic enzymes was not examined and remains unclear.

Nitrate ion may play a special role during anoxia, by acting possibly as a terminal electron acceptor in respiration in the absence of molecular O_2, with NADH-dependent reduction of nitrate to nitrite via NR in 'nitrate respiration' (7, 12, 18). It's significance lies in the regeneration of NAD^+, essential for the continuation of glycolysis. Additionally, it has been suggested that competition for cytoplasmic pools of NADH between NR and ADH can occur, thereby lowering the rate of ethanol production (7, 12). According to one hypothesis (12), improved cell survival should result from limiting the accumulation of ethanol which is assumed to be toxic. If nitrate reduction effectively recycles NAD^+, and this assists the flow of carbon through glycolysis, with it's substrate-linked phosphorylations, such

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[2] Abbreviations: ADH, alcohol dehydrogenase; AEC, adenylate energy charge; HPT, hypoxically pretreated; NHPT, not hypoxically pretreated; NR, nitrate reductase.

changes should reflect in higher levels of energy metabolism. In accordance with this hypothesis, the ratio $NAD^+/NADH$ was raised when rice root apices in anaerobic media were supplied with 7 mM nitrate, and there was an increase (from 0.2–0.35) in AEC after 24 h anaerobiosis (18, 19).

In the present paper we examine the energy relations and ethanolic fermentation of anoxic maize root tip cells that had previously been hypoxic (exposed to 2–4 kPa O_2 partial pressure) or had been suddenly exposed to anaerobic conditions. We show that hypoxia induced a change in metabolism that improved the energy relations of anoxic cells, and contributed to a more prolonged survival in the absence of O_2. We also show that the presence of nitrate ions failed to modify the energy relations of root tips during anoxia.

MATERIALS AND METHODS

Plant Material. Maize (*Zea mays* L. cv INRA 508) was germinated in the dark at 25°C on wet filter paper. At 3 d from imbibition, germinated seedlings were transferred to 10 L volumes of either 0.5 mM $CaCl_2$ or 0.5 mM $Ca(NO_3)_2$ in 20 L-capacity rectangular-shaped tanks. Seedlings, 50 to 100 per tank, were inserted individually through holes in an expanded polystyrene 'raft' which floated on the surface of the solution. Aeration was initially from air pumped through sintered glass blocks placed at the bottom of each tank. After 4 h the concentration of $Ca(NO_3)_2$ was raised to 5.0 mM, and that of $CaCl_2$ also to 5.0 mM, (or in some experiments, to 2.5 mM $CaCl_2$ with 2.5 mM $CaSO_4$). All experiments were carried out in a dark room at 25°C.

Aeration Pretreatment. About 1 h after raising the concentration of calcium salts, solutions were vigorously bubbled (at about 30 L h^{-1}) with premixed, compressed gas volumes of either 40 to 50% (v/v) O_2 in N_2, well in excess of the critical O_2 pressure at 25°C (27) or with 2 to 4% (v/v) O_2 in N_2 (see Tables), equivalent to O_2 partial pressures (pO_2) of 2 to 4 kPa, approximately one-tenth to one-fifth of that in ambient air (pO_2 = 20.6 kPa). Pretreatments continued for 18 h, the tops of the tanks being sealed with plastic film to maintain the same gaseous atmosphere around shoots as roots, to avoid internal O_2 movement from shoots to roots. To follow the extension of roots during this time, each was marked 10 mm behind the tip with a spot of carbon (charcoal slurry) at the start of the pretreatment period, and measured again at the end of the treatment. In some experiments we used maize that was homozygous for a mutation at the Adh1 locus and produced little, if any, enzymatically active ADH1 (21).

Anaerobic Treatment. Root apices, 5 mm long, were excised and placed in groups of 10 in 10 ml-volume glass vials with rubber puncture caps in 2.0 ml of the solution in which the roots had been pretreated, but supplemented with 100 mM glucose. Hypodermic needles through the rubber caps were used to gas the solution and headspace, one needle bubbling gas into the solution, the other to give gas exit from the vial. Equilibrium between these small volumes and a new gaseous atmosphere was attained in a few minutes. Roots from the fully aerobic and the hypoxic pretreatments were gassed either with 40 to 50% (v/v) O_2 in N_2, or with O_2-free N_2 (the anaerobic treatment, N_2 = 99.99%). There was 3-fold replication.

Extraction and Estimation of Adenine Nucleotides. Solution was forced out from each vial by the gas mixture without any contamination from air, by pushing the tip of the 'exit' needle into the solution at the bottom of the vial. The roots were frozen *in situ* with liquid N_2. In some experiments, roots were reoxygenated by bubbling with air for 30 min before freezing. The tissue was stored at −80°C, lyophilized at −30°C, and nucleotides were extracted for ATP, ADP, and AMP estimation using published procedures (26). Briefly, lyophilized roots were homogenized at −20°C in 0.6 M TCA in diethyl ether, then in aqueous TCA. The TCA was removed by partitioning into diethyl ether, the pH of the aqueous phase was adjusted to neutrality with NaOH, and ATP was assayed by a luciferin-luciferase reaction. Enzymes were used to convert ADP and AMP to ATP for assay, and internal standards were included throughout.

Nitrate Reductase Activity during Anoxia. Batches of 20 root apices 5 mm long, were incubated in 2.0 ml of 5 mM $(Ca(NO_3)_2$ with 100 mM glucose and bubbled with 50% oxygen in nitrogen in 10 ml-volume glass vials. After 30 min, vials were flushed with O_2-free N_2 gas for 30, 60, or 90 min and tissues were killed by 5 min boiling. Assay for NO_2^- in the medium was done colorimetrically by reacting 500 μl of the medium with 250 μl of sulfonilamide (10 g L^{-1} in 3 N HCl) and 250 μl of *N*-1-naphthyl-ethylene-diamine-dichloride (0.2 g L^{-1}) for 15 min. Color was read in a spectrophotometer at 540 nm.

Alcohol Dehydrogenase Activity. For *in vivo* estimation, batches of 10 excised root apices were incubated in 10 ml-volume glass vials fitted with rubber puncture caps in 2.0 ml volumes of solution of the same composition as that in which they had been grown, but containing 100 mM glucose. Nitrogen gas was vigorously passed through the vials for 5 min using hypodermic needles, then the vials were sealed and continuously shaken. Ethanol was analyzed by withdrawing 2 μl volumes using a microsyringe and injecting into a gas chromatograph. The 2 m column was packed with 'Poropak QS,' with helium as carrier gas (25 ml min^{-1}), isothermal 160°C conditions, and flame ionization detector. For *in vitro* estimation, five root apices (5 mm) were homogenized in 200 μl of 10 mM tris buffer containing 5 mM DTT (pH 7.4) and 0°C. The reaction mixture comprised: 100 μl of glycine buffer (pH 9.0), 20 μl of 10 mM NAD, 100 μl of 1 M ethanol, the required volume of plant extract (10 μl) together with distilled water to make a final volume of 780 μl. Volumes of 5, 10, 15, and 20 μl of extract gave ADH activities that were proportional to the volumes of extract. Protein in the extracts was determined by the method of Bradford (6).

Gel Electrophoresis. The above plant extracts were also assayed for ADH isozymes by electrophoresis on native polyacrylamide gels, using about 40 μg of protein extract per lane. Isozymes were stained and identified by published procedures (9–11).

RESULTS

Energy Metabolism. After 1 h without oxygen, the ATP content of NHPT root tips was greatly lowered. There was a concomitant rise in AMP, and little change in the total adenine nucleotides (Table I). The ratio of ATP/ADP and the AEC were both greatly lowered.

For HPT roots, the anaerobic period also lowered the ATP content, but the effect was a little less than in NHPT roots. The ATP/ADP ratio, and particularly the AEC, were maintained at higher levels in HPT roots. Pretreatment with 2.3% O_2 was not itself irreversibly damaging: on exposure to 46% O_2, apices showed an energy metabolism comparable to that in those continuously maintained at the higher concentration of O_2 (Table I). During the hypoxic pretreatment roots extended more slowly (0.82 mm h^{-1}) but returned to a rate of 1.59 mm h^{-1} when bubbled again with air, a rate that was similar to fully aerobic controls. Roots exposed to NO_3^- during anoxia had very similar ratios of ATP/ADP and AEC to those of roots kept in NO_3^--free media, both for NHPT and HPT tissues.

The effect of a hypoxic pretreatment and the presence of NO_3^- on energy metabolism in root apices was determined over longer periods of anoxia, followed by 30 min reoxygenation (40% O_2 [v/v]) before extraction of nucleotides. Compared with controls maintained at 40% O_2, the amount of ATP per tip in NHPT roots greatly declined after 7 h of anoxia (Fig. 1A). Following 24

Table I. *Effect of Hypoxic Pretreatment and Presence of Nitrate or Chloride on Energy Metabolism in Excised Root Apices in Zea mays*

Intact plants were pretreated for 18 h with roots in either 2.5 mM $CaCl_2$ + 2.5 mM $CaSO_4$ or 5.0 mM $Ca(NO_3)_2$ and bubbled with either 46% or 3.1% O_2 in N_2. Excised root tips were incubated for 1 h in the same solutions, with 100 mM glucose, and gassed with either 46% O_2 (aerobic) or O_2-free N_2 gas (anaerobic).

Treatment	ATP	ADP	AMP	Total Adenine Nucleotides	ATP/ADP	AEC
	pmol per 5 mm root apex				*ratio*	
Not hypoxically pretreated						
Aerobic + Cl^-	966	264	24	1254	3.93	0.877
Aerobic + NO_3^-	1198	199	0.4	1397	6.11	0.929
Anaerobic + Cl^-	296	342	371	1009	0.86	0.463
Anaerobic + NO_3^-	234	317	513	1064	0.72	0.363
Hypoxically pretreated						
Aerobic + Cl^-	668	124	6	798	5.42	0.914
Aerobic + NO_3^-	988	178	5	1171	5.75	0.920
Anaerobic + Cl^-	295	223	57	575	1.33	0.715
Anaerobic + NO_3^-	388	362	253	1003	1.08	0.569
LSD ($P < 0.05$) for treatments	186	87	81	272	1.26	0.060

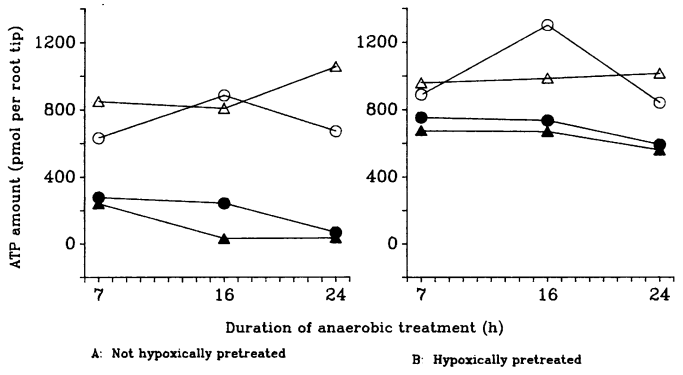

FIG. 1. Effect of duration of anoxia on amounts of ATP in root apices. Roots were either hypoxically pretreated by gassing for 18 h with 3% O_2 in N_2, or not hypoxically pretreated by gassing with 40% O_2 in N_2, before excision of 5 mm apical segments. Excised root tips were incubated for the stated times under anaerobic conditions or in 40% oxygen with either $CaCl_2$ + $CaSO_4$ or $Ca(NO_3)_2$ solutions containing 100 mM glucose. Roots were reexposed to O_2 (40%) for 30 min before extraction of adenine nucleotides. Treatments were: (○), Cl^- + 40% O_2; (●), Cl^- + N_2; (△), NO_3^- + 40% O_2; (▲), NO_3^- + N_2.

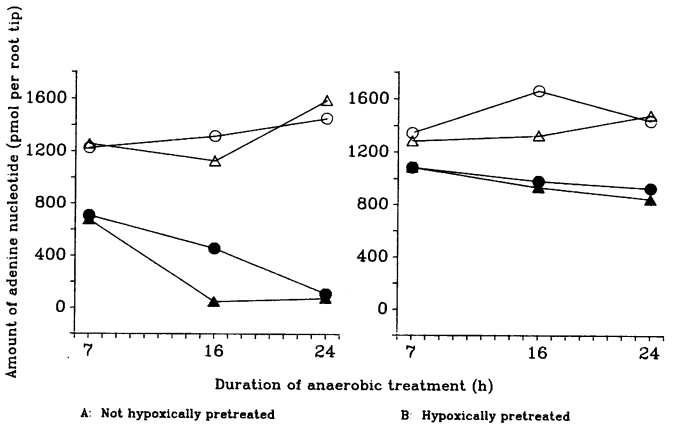

FIG. 2. Effect of duration of anoxia on amounts of adenine nucleotides in root apices. Conditions were as described for Figure 1.

h of anoxia, ATP was only about 5% that of controls. This contrasts strikingly with the response of HPT roots where ATP gave only a small decline with time under anoxia and at 24 h was about 70% of aerobic controls (Fig. 1B).

With increasing duration of exposure to anoxia, total amounts of adenine nucleotides had already declined sharply at 7 h in NHPT roots (Fig. 2A), and the trend continued up to 24 h. Data for ADP and AMP are not shown separately, but both declined at a similar rate to that given in Figure 2A. Total nucleotides in the HPT tissues during anoxia were much more stable with time (Fig. 2B), with concentrations intermediate between those of aerobic controls and NHPT anoxic cells. In NHPT cells, values for the AEC (Fig. 3A) were anomalous because of the parallel decline in ATP and the simultaneous degradation of other adenine nucleotides. The fact that the root tips were flaccid indicates they were probably dead (21). For HPT roots (Fig. 3B) in which the total nucleotides remained essentially stable, the AEC was restored on reoxygenation to a value equal to that of aerobic controls. At 24 h, root apices in 40% O_2 had extended from their initial 5 mm length to 10 mm, but under anaerobic conditions there was no cell extension. However, all HPT root tips maintained their turgidity under anoxia.

Nitrate Reduction and ADH Activity. The rate of reduction of nitrate to nitrite under anaerobic conditions was estimated from the release of nitrite to the medium. Work elsewhere shows clearly that nitrate reduction is slow in anoxic roots and that nitrite leaks readily and almost quantitatively to the outer solution (14). We found an appreciable release of nitrite from anoxic

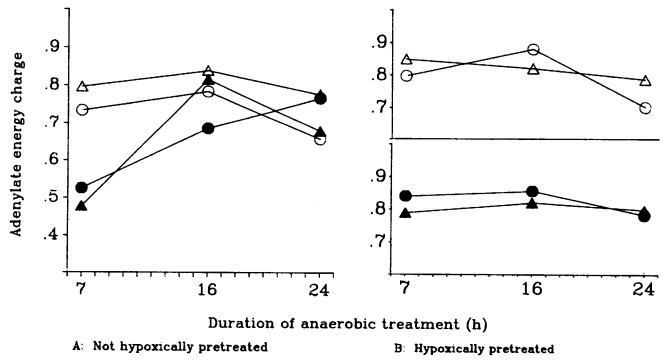

FIG. 3. Effect of duration of anoxia on adenylate energy charge in root apices. Conditions were as described in Figure 1.

roots incubated with 5 mM $Ca(NO_3)_2$ (Fig. 4), with no difference between HPT and NHPT roots: the average reduction rate was 2.0 nmol h^{-1} per root. As expected, because NR is substrate induced, there was no appreciable nitrite production by roots that had been grown on nitrate-free $CaCl_2$ and then incubated with $Ca(NO_3)_2$ (data not presented).

ADH activity in extracts showed no difference in activity between Cl^- or NO_3^- treated roots (Table II). For both treatments, there was approximately a 15-fold increase in ADH activity in HPT roots compared with NHPT ones. This difference in ADH activity, expressed per mg protein, was not the result of breakdown of protein at the low concentration of O_2 used to bring about hypoxia: protein content per apex was slightly greater in HPT roots.

Energy Metabolism, ADH Activity and Ethanol Production in Adh1⁻ Mutants. After 1 h of anaerobic treatment, there was a marked contrast between NHPT and HPT roots (Table III). There was appreciably greater energy metabolism in HPT roots, comparable to that in nonmutant roots (Table I). Following 9 h anaerobic treatment, NHPT roots failed to respond to reoxygenation and energy metabolism remained at a low level with loss of turgor (Table III), suggesting cell death had occurred. With HPT roots, energy metabolism was restored by the reintroduction of oxygen to a level similar to that of fully aerobic tissues (*cf.* Table I, Figs. 1 and 2). Even at 22 h anaerobic treatment, HPT roots of Adh1⁻ mutants showed a high level of energy metabolism when reoxygenated, and appeared turgid (Table III).

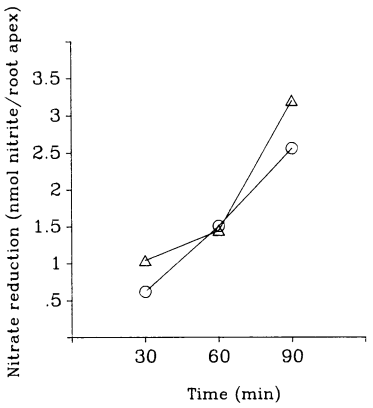

FIG. 4. Nitrate reductase activity *in vivo* of anoxic roots. Roots of plants grown in $Ca(NO_3)_2$ solution were excised and incubated anaerobically with 5 mM $Ca(NO_3)_2$ and 100 mM glucose. (Δ), hypoxically pretreated; O, not hypoxically treated.

Table II. *ADH Activity in Root Extracts*

Intact plants were pretreated for 18 h with roots in either 5.0 mM $CaCl_2$ or 5.0 mM $Ca(NO_3)_2$ and bubbled with either 50% or 2.3% O_2 in N_2. Excised root tips were extracted and assayed for ADH, as described in "Materials and Methods."

Treatment	Protein Content of Extract	ADH-Activity	HPT/NHPT
	μg per root apex	*(μmol min^{-1} mg^{-1} protein) $\times 10^3$*	*ratio*
Not hypoxically pretreated			
Cl^--treated	152	18.8	
NO_3^--treated	180	19.0	
Hypoxically pretreated			
Cl^--treated	248	289	15.4
NO_3^--treated	226	298	15.7

Thus, hypoxically pretreated Adh1⁻ mutants behaved very similarly to the hypoxically pretreated nonmutants.

ADH activity in NHPT roots was low, that in the Adh1⁻ mutants being only 16% that in INRA 508 (Table IV). Following HPT, roots of mutant plants showed higher levels of ADH activity, but INRA 508 increased even more, to 14 times its uninduced level. HPT also led to greater rates of production of ethanol by mutant and nonmutant roots (Table IV). However, it is noticeable that this *in vivo* production did not correlate well with ADH activity. Assuming an average protein content of 200 μg per root tip, *in vivo* production was between 8 and 140 times less than ADH activity (*cf.* Table II). This consideration does not detract from the general relation between ADH activity (Table II), ethanol production (Table IV), and energy metabolism (Table III) in both genotypes.

Gel Electrophoresis. Hypoxic pretreatment of INRA 508 roots was associated with a pronounced increase in staining bands for ADH1·ADH1 homodimers and also ADH1·ADH2 heterodimers (Fig. 5), lane 2. Homodimers of ADH2·ADH2 were detected on the originals as faint bands. In extracts from the roots of induced Adh1⁻ mutants, ADH2·ADH2 could be identified (lane 4), accounting for the ADH activity in extracts determined above (Table IV), but ADH1 isozymes were barely detectable.

DISCUSSION

Energy Metabolism. In earlier studies we showed that aerenchyma formation in maize roots is stimulated by hypoxia, the internal transport of oxygen (originated from the leaves) leading to an improved energy status of cells in the root tip in an anaerobic medium (8). Such structural changes cannot explain the present results, because the use of excised tissue during the anaerobic treatment eliminated any possibility of internal oxygen transport. It is clear that during an 18 h hypoxic pretreatment, changes in the metabolism of root tip cells must take place that improve their tolerance of a subsequent period under totally anaerobic conditions. Assay of the amounts of ATP, as well as the ratios ATP/ADP and AEC, indicators of the limitation of metabolic activity by respiring cells (17), all point to an appreciable maintenance of energy metabolism under anoxia following a hypoxic pretreatment (Table I).

The visible loss of turgor in NHPT root tips after 7 h of anoxia, even in the presence of glucose, reflects a lack of osmotic competence of plasma membranes, a situation which presumably cannot be sustained for long by viable cells. Its association with low levels of total adenine nucleotides (Fig. 2), even after reoxygenation, indicates a loss of viability which clearly contrasts with the ability of HPT root tips to survive up to 22 h anoxia. The loss of total nucleotides may be symptomatic of cell death, rather than a regulation of total adenylate pool size by AMP deaminase (28).

The retention of turgor by HPT cells during anoxia is interesting, because it indicates that although the restoration of energy status in these cells is only part way towards that in fully aerobic ones, it is sufficient for the maintenance of integrity of plasma membrane and/or tonoplast.

Nitrate Respiration. We failed to find reliable evidence of a positive effect of nitrate on energy metabolism, irrespective of whether roots had been hypoxically pretreated or not. This contrasts with the results of Reggiani *et al.* (18, 19) who found that in roots of rice (rice roots are anoxia-intolerance, like those of maize), 7 mM NO_3^- gave a greater AEC and also increased the ratio NAD/NADH, which would be expected if NR activity speeded up the regeneration of NAD. Several aspects of their paper cause us to question it. First, the effect of NO_3^- on AEC was small, there was no statistical indication of experimental error, and there were no data on the concentrations of ATP, ADP, or AMP, so that we cannot determine whether analyses

Table III. *Effect of Hypoxic Pretreatment on Energy Metabolism and ADH Activity in Excised Root Apices of Zea mays Adh1⁻ Mutants*

Nucleotides are in pmol per root apex. Intact plants were pretreated by exposing roots to 5.0 mM $Ca(NO_3)_2$ solution bubbled with air (NHPT) or with 3.2% O_2 for N_2 in 48 h HPT. Excised root apices (5 mm) were made anaerobic by gassing with O_2-free N_2 gas for the stated times. Tissues were then either extracted for nucleotides, or bubbled with air for 30 min before extraction. Root tips were either flaccid (f) or turgid (t) at the end of the experiment.

Treatment	ATP	ADP	AMP	Total Adenine Nucleotides	ATP/ADP	AEC
	pmol per root apex				*ratio*	
Anaerobic (1 h)						
NHPT	155	291	612	1059	0.58	0.28t
HPT	410	276	159	846	1.49	0.65t
Anaerobic (9 h) then reoxygenated						
NHPT	51	136	29	216	0.38	0.55f
HPT	786	130	0	926	6.1	0.93t
Anaerobic (22 h) then reoxygenated						
HPT	915	143	0	1058	6.4	0.93t
LSD ($P < 0.05$)	202	73	6	235	2.0	0.19

Table IV. *ADH Activity in Root Extracts and Ethanol Production Rates in Vivo in Root Tips of Zea mays*

Intact plants were pretreated for 18 h with roots in 5.0 mM $Ca(NO_3)_2$ and bubbled with either 50% or 2.3% O_2 in N_2. Excised root tips were assayed for ADH activity in extracts, or for ethanol production during anaerobic incubation as described in "Materials and Methods."

Treatment and Genotype	Alcohol Dehydrogenase Activity	HPT/NHPT	Ethanol Production Rate	HPT/NHPT
	μmol min⁻¹ mg⁻¹ protein ×10³	*ratio*	*nmol h⁻¹ per tip*	*ratio*
	mean ± SE			
Not hypoxically pretreated (NHPT)				
INRA 508	67 ± 8 (n = 8)		25	
Adh1⁻ mutant	11 ± 2 (n = 7)		17	
Hypoxically pretreated (HPT)				
INRA 508	927 ± 248 (n = 3)	14	80	3.2
Adh1⁻ mutant	80 ± 5 (n = 16)	7	69	2.8

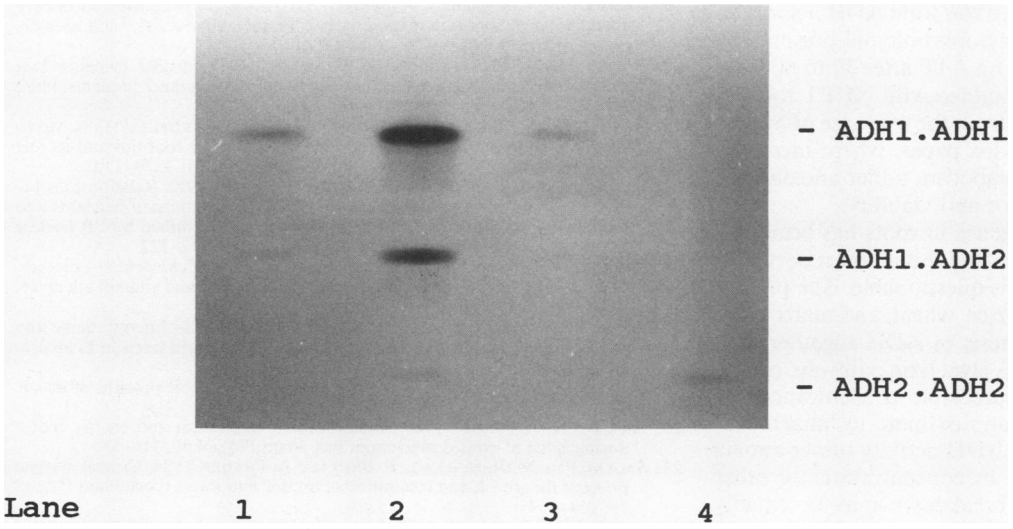

FIG. 5. Effect of hypoxic pretreatment on ADH isozymes in maize root tips. Isozymes were separated by native polyacrylamide gel electrophoresis. The lanes were: 1, NHPT, INRA 508 (35 mg protein); 2, HPT INRA 508 (39 mg protein); 3, NHPT Adh1⁻ mutant (41 mg protein); 4, HPT Adh1⁻ mutant (42 mg protein).

were of cells in which total nucleotides had declined (compare this paper, Fig. 3). Second, extraction of adenine nucleotides as well as pyridine nucleotides was in 0.6 M $HCLO_4$ (18), yet it is well known that NADH and NADPH are rapidly destroyed at acid pH: according to Lowry and Passonneau (15), NADH is 99% destroyed at pH 2 at 23°C in 1.2 min, and we measured similar rates of destruction in our own laboratory. Evidence supporting anaerobic nitrate reduction in maize roots comes from ^{31}P-NMR studies of intracellular pH (20). The development of cytoplasmic acidosis, a determinant in cell death in anaero-

bically treated, excised root tips, was retarded when cells where exposed to 25 mM $Ca(NO_3)_2$. The authors speculated that NO_3^- might be acting by reoxidizing cytoplasmic and mitochondrial NADH, thereby allowing continued operation of the TCA cycle, and at the same time diverting NADH from lactic acid synthesis via lactate dehydrogenase. However, no direct evidence as to the role of NO_3^- was presented; neither was the specificity of NO_3^- as compared with Ca^{2+} tested.

Comparison of the relative rate of nitrate reduction (Fig. 4) and ethanol production (Table IV), noting that either reductive step would regenerate 1 mol of NAD, weakens the case for nitrate reduction as an effective means of NAD regeneration under anaerobic conditions. In maize root tips, the anerobic reduction of NO_3^- to NO_2^- (Fig. 4) was about 10% the rate of reduction of acetaldehyde to ethanol (mol per root tip) in uninduced roots (Table IV) and only 3% that in induced ones; similar rates of nitrate reduction (per g fresh weight) were measured in anoxic barley roots (14). It thus seems highly unlikely that NR and ADH effectively compete for NADH. Furthermore, the presence of NO_3^- did not depress the rate of ethanol production *in vivo* (data not presented) or affect ADH activity in extracts (Table II). While there is no doubt that nitrate-nitrogen assists the tolerance or recovery of plants in flooded soil (13) the explanation may lie more in the requirement of combined N as a substrate for growth, than by acting specifically in relation to anaerobic respiration (29).

Energy Metabolism and Alcohol Dehydrogenase. A major question to be resolved is the role of ADH during anoxia. In maize root tips, ethanol and CO_2 are the main products of anaerobic metabolism, with smaller amounts of lactate (21, 28). The rate of production of ethanol and lactate is quantitatively related to the AEC under these conditions (28), which is to be expected if substrate-linked phosphorylations in glycolysis, together with regeneration of NAD in production of ethanol and lactate, account for ATP synthesis. Hypoxia evidently induces greater activity of ADH1 and ADH2 in INRA 508, and induces ADH2 in the $Adh1^-$ mutant (Table IV; Fig. 5).

Our observations on acclimation to anoxia in roots of $Adh1^-$ mutants contrast with those of Roberts *et al.* (21) who likewise treated excised roots with O_2-free N_2 gas (described as a hypoxic treatment in their paper, but equivalent to an anaerobic or anoxic treatment in ours). With anoxia, there was little ADH activity in their roots, a continuous decline in cytoplasmic pH presumably due to lactate synthesis, and little or no ATP after 30 to 60 min. These results agree well with ours obtained with NHPT roots of $Adh1^-$ mutants, but differ markedly from the response of hypoxically pretreated ones described in this paper, where increased ADH activity and greater energy metabolism under anoxia were associated with maintenance of turgor and viability.

Induction of the activity of Adh genes in roots has been well characterized (9–11, 25), but whether the induction reported in earlier papers was strictly anaerobic is questionable. The present work, together with other reports on rice, wheat, and maize roots (3, 4, 30), makes it clear that synthesis of ADH together with increased amounts of enzymes of the glycolytic pathway, can be induced by media with low but appreciable concentrations of O_2, conditions which do not even approximate to 'anaerobic.' We do not know whether increased ADH activity under anoxia has a special significance. Increases in concentrations of other enzymes involved in carbohydrate breakdown may be equally important; pyruvate decarboxylase is much more likely to be limiting to ethanol formation in maize roots than ADH (30). Nevertheless, we conclude that 'hypoxic training' in maize root tips, which confers an improved energy status and tolerance of anaerobic conditions, is closely linked to induction of an effective ethanolic fermentation pathway.

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