Induction of Lactate Dehydrogenase Isozymes by Oxygen Deficit in Barley Root Tissue

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ABSTRACT
Lactate dehydrogenase (LDH) activity in attached roots of barley and other cereals increased up to 20-fold during several days of severe hypoxia, reaching a maximum of about 2 micromoles per minute per gram fresh weight. In barley, induction of LDH activity was significant at 2.6% O2 and greatest at 0.06%, the lowest O2 concentration tested. Upon return to aerobic conditions, induced LDH activity declined with an apparent half-life of 2 days. The isozyme profile of barley LDH comprised 5 bands, consistent with a tetrameric enzyme with subunits encoded by two different LDH genes. Changes in staining intensity of the isozymes as a function of O2 level suggested that one LDH gene was preferentially expressed in severe hypoxia. When tracer [U-14C]glucose was supplied to induced roots under hypoxic conditions, lactate acquired label, but much less than either ethanol or alanine. Most of the [14C]lactate was secreted into the medium, whereas most other labeled anionic products were retained in the root. Neither hypoxic induction of LDH, nor lactate secretion by induced roots, is predicted from the Davies-Roberts hypothesis, which holds that lactate glycolysis ceases soon after the onset of hypoxia due to acidosis brought about by lactate accumulation in the cytoplasm. These results imply a functional significance for LDH beyond that assigned in this hypothesis.

Flooding creates anoxic soil conditions within 1 to 2 d (6). Such conditions preclude aerobic respiration so that root survival becomes dependent upon fermentative metabolism, of which the best characterized pathway is ethanol glycolysis (3). A change in the pattern of gene expression takes place in roots deprived of O2; in maize (22) and other cereals (9) the synthesis of certain proteins is induced. These anaerobic proteins include ADH2 (22), PDC (16), and two other glycolytic enzymes (13, 14), which implies a need to enhance or maintain the capacity for ethanol glycolysis during O2 deficit. Direct evidence for the importance of ADH and ethanol glycolysis during hypoxia comes from Adh1 null mutants of maize (23) and barley (10); these mutants are abnormally susceptible to flooding at germination.

A potential alternative to ethanol as a glycolytic end product is lactate, produced via pyruvate reduction catalyzed by LDH (Fig. 1). Relatively little is known about either LDH or the lactate branch of glycolysis in plants, particularly in relation to chronic O2 deficiency. Although LDH occurs in diverse plant tissues, often in the form of multiple isozymes (18), some reports suggest that LDH is absent from anaerobic roots (16). On the other hand, 2- to 4-fold anaerobic induction of LDH activity has been found in roots of two marsh plants (25) and in barley aleurone layers (8). In animals, lactate formation is a principal means of NAD regeneration during prolonged hypoxia (5), but it is unclear whether this is so in plants. Lactate glycolysis can certainly take place in anaerobic roots (24) and in germinating seeds (1, 15), although possibly only as a transient burst immediately after transfer from aerobic to anaerobic conditions (4). Indeed, Davies et al. (4) have proposed that a brief, self-limiting phase of lactate accumulation serves to lower cytoplasmic pH and thereby activate PDC and ethanol glycolysis in anoxic roots. The in vivo NMR data of Roberts et al. (21) for maize root tips support this concept.

In this work we set out to determine whether the roots of barley and other cereals possess constitutive or anaerobically inducible LDH isozymes, and to assess the glycolytic flux to lactate in anaerobically induced barley roots.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Barley (Hordeum vulgare L., cv Himalaya) seeds were surface-sterilized in 1% NaOCl (w/v) for 20 min, rinsed with water, and planted in vermiculite. After 4 d growth in the dark at room temperature, individual seedlings were inserted through the hole of a rubber stopper and secured with a foam rubber plug. Thirty such plants were set in a Plexiglas board cut to fit a 20-L fish tank. Plants were grown hydroponically with roots in darkness in half-strength Hoagland solution sparged continuously with air for 10 to 20 d. Hypoxic conditions were achieved by sparging with N2 or N2 mixed with air. The nutrient solution was changed 10 d after transplanting. Growth chamber conditions were: day, 16 h, 21°C, 100 μmol photon m⁻² s⁻¹ photosynthetic photon flux density, vapor pressure deficit 5 hr; night, 8 h, 16°C. Other cereals (Zea mays cv W22, Triticum aestivum cv Chinese Spring; Secale cereale) were grown in the same way.

For experiments requiring aseptically grown roots, seeds were shaken for 10 min in a sterile beaker containing 70% ethanol, for 15 min in commercial bleach (5.25% NaOCl, w/v) containing 0.5% (v/v) Tween 20, and were rinsed in sterile distilled H2O. Three seeds were germinated per 10-cm Petri dish containing nutrient broth agar (Difco). After 3 d in the dark and 2 d in light, seedlings showing no microbial contamination were transferred to individual foil-wrapped, sterile 250-ml Erlenmeyer flasks containing sterile half-strength Hoagland solution. Seedlings were supported in split foam rubber stoppers which were in turn inserted into holes in rubber stoppers which fitted into the flasks. Into each stopper were inserted two 16-gauge needles. One needle was connected externally to an air line via a bacterial filter (Gelman Sciences) and internally to an airstone. The other needle

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3. Abbreviations: ADH, alcohol dehydrogenase; LDH, lactate dehydrogenase; PDC, pyruvate decarboxylase.
Fig. 1. Lactate and ethanol glycolysis.

was wrapped in foil and was used for replenishing the nutrient solution. Seedlings were grown in a laminar flow hood at 20°C, under continuous light. Seedlings, 150 μmol photon m⁻² s⁻¹ photosynthetic photon flux density, for 10 d while sparging with humidified air and for a further 5 d with humidified N₂.

**Extraction and Assay of LDH.** Extractions were carried out at ice temperature. Roots were rinsed in distilled H₂O, briefly blotted, weighed and ground with sand in a mortar and pestle in 0.1 M Tris-HCl (pH 8.5) containing 10 mM Na borate, 10 mM DTT, 5 mg/ml BSA, and 15% (v/v) glycerol (2 ml/g roots). The brei was centrifuged for 2 min in a microfuge and aliquots of the supernatant were taken for enzyme assay. LDH was assayed spectrophotometrically in the pyruvate → lactate direction by monitoring pyruvate-dependent NADH oxidation at 340 nm in the presence of 4-methylpyrazole to inhibit ADH, and NaCN to lower the background rate of NADH oxidation. The assay mix (final volume 1.3 ml) contained 1 ml of 0.13 M Tris-HCl (pH 8.0), 150 μg NADH, 3 μmol 4-methylpyrazole, 3 μmol NaCN, 15 μmol Na pyruvate, and 0.2 ml of enzyme extract. LDH activities are reported in IU (μmol/min).

**Extraction and Assay of Lactate.** Roots were harvested, quickly blotted, and immediately placed in liquid N₂. Frozen roots were weighed and ground in 5% (w/v) HClO₄ (2-4 ml/g roots) containing a spike (e.g. 20 nCi) of L-[¹⁴C]lactate (Amer sham, 161 nCi/μmol) for isozone dilution analysis. After standing for 20 min at 0°C the brei was centrifuged; the supernatant was decanted and neutralized with KOH. Extracts were separated by ion exchange essentially according to Reilly (19). Dowex-1 (OH) columns (1 ml bed volume, 200-400 mesh) were converted to the acetate form by washing with 6 ml of 1 M Na acetate followed by 4 ml of water. Samples (3 ml) were then loaded and eluted with 6 ml of 0.5 M HCOOH. Fractions were collected and assayed for ¹⁴C content by scintillation counting. Lactate eluted between 2 and 5 ml. The lactate fraction was evaporated to dryness at 30°C under a stream of N₂, redissolved in 50% ethanol, and applied to Whatman 3MM paper strips (57 × 5 cm) for descending chromatography and developed for 10 h with ethanol: NH₄OH: water (80:5:15, v/v). The lactate zone was located by radioautography, cut out, and eluted with 4 × 0.2 ml water washes. Portions of the eluate were taken for enzymic assay of lactate (7) and for ¹⁴C determinations to adjust for recovery. All materials for lactate assay were handled with gloves to avoid contamination from sweat.

**¹⁴C Glucose Experiments.** Aseptically-grown roots were fed labeled glucose under sterile conditions. A weighed portion of each root was ground in sterile water, serially diluted, and plated on nutrient broth agar to check bacterial contamination. Only data for roots with <10⁶ colony forming units/g are reported. Root samples (0.2-0.5 g) were placed in 250-ml flasks containing 2 ml of half-strength Hoagland solution. A well containing 0.2 ml of 10 M KOH and a filter paper wick was suspended in each flask to trap ¹⁴CO₂. Flasks were sealed with a rubber stopper, flushed with humidified and filtered O₂-free N₂ for 15 min, and then the desired amount of O₂ was injected through a serum cap inserted in a port in the rubber stopper. δ-[¹⁴C]Glucose (2 μCi, 240 μCi/μmol, Amersham) in 2 ml of half-strength Hoagland solution was then injected, and the flasks were incubated for 8 h in darkness at 25°C at 150 rpm on a gyratory shaker. The O₂ concentration was measured at the beginning and end of the incubation. At the end of the incubation, the medium was drawn off and the roots were swirled for 1 min in 5 ml of water which was combined with the original medium. To the roots were added 20 ml of 80% ethanol; 45 ml of 95% ethanol was added to the incubation medium. Samples were held at 4°C for 24 to 48 h before analysis.

**Analysis of Labeled Compounds.** ¹⁴CO₂ trapped in the KOH wick was eluted with 4 x 0.5 ml of water; a 10-μl aliquot of the eluate was placed on a 1 x 1 cm square of filter paper impregnated with 50 μl of saturated Ba(OH)₂ solution, and dried in an air stream. The paper squares were placed in 25-ml scintillation vials with 5 ml of scintillation fluid (Safety Solve, RPI) and counted in a Beckman LS 3801 scintillation counter. The counting efficiency of this system was 79%. Radioactivity in insoluble material was determined after combustion in a Packard Tricarb Sample Oxidizer.

Root extract and medium samples received carrier glucose (5 mg) and carrier Li lactate (2 mg) prior to extraction. Flasks containing the samples were connected to a condenser and were heated in a boiling water bath; about 3 ml of distillate were collected. A sample of distillate was taken for [¹⁴C]ethanol estimation by scintillation counting; an equal volume was counted in the Ba(OH)₂-filter paper system described above, to correct for any ¹⁴CO₂ or [¹⁴C]lactate present. Liquor remaining in the flask was drawn off, and in the case of root samples the tissue was reextracted by steeping in 10 ml of 80% ethanol at room temperature for 30 min; this extract was pooled with the first. Samples were concentrated in vacuo at 30°C and separated into cationic, anionic, and neutral fractions as described (8). Fractions were reduced to dryness in vacuo at 30°C, and analyzed further by electrophoresis and TLC. Systems used were: electrophoresis on 0.1 mm cellulose plates in 70 mm Na borate (cation fraction); TLC on 0.25 mm cellulose plates developed with 1-butanol:acetic acid:water, 60:15:25, v/v (anion and cation fractions), or ethanol: NH₄OH: water, 8:2:1, v/v (anion fraction), or on 0.1 mm cellulose plates with 1-propanol:ethyl acetate:water, 7:1:2, v/v (neutral fraction).

Radioactive zones were detected by autoradiography. Organic acids were detected with an indicator spray (0.05% bromophenol blue in 0.2% citric acid) and amino acids with 0.25% ninhydrin in acetone. Identities of labeled compounds were based on comigration with authentic standards in two of the systems above. The ¹⁴C-content of individual compounds were determined by scintillation counting after scraping radioactive zones from plates and eluting with water.

**Polyacrylamide Gel Electrophoresis.** Nondenaturing electrophoresis was performed in slab gels (1.5 mm thickness) as follows. The running gel contained (w/v) 8.6% acrylamide-0.11% bisacrylamide and 0.43 mM Tris-HCl (pH 8.4) and was polymerized with 0.92 μl/ml N,N,N',N'' tetramethyl ethylenediamine (TEMED) and 0.41 mg/ml ammonium persulfate. The stacking gel was 7.5% acrylamide-0.1% bisacrylamide, 60 mM Tris-HCl (pH 6.7), 20% (w/v) sucrose, 0.6 μl/ml TEMED, and 5 μg/ml riboflavin, photopolymerized with a fluorescent tube overnight. Gels were run for 28 h at 4°C at 200 V in a Hoefer SE-600 unit using 5 mm Tris-38 mm glycine running buffer. Gels were stained for LDH activity as described (8).

**Gas Measurements.** O₂ in solutions was measured with a Yellow Springs model 53 oxygen monitor connected to a chart recorder using aerated distilled H₂O as a standard. Data are expressed as an equivalent percentage of O₂ in the atmosphere. O₂ levels in head space samples were determined by GC in 0.5-ml samples withdrawn with a syringe. A Varian 3700 gas chromatograph equipped with a thermal conductivity detector and a stainless steel column packed with molecular sieve 5A, 45 to 60
RESULTS

Induction of LDH Activity. Extraction of anaerobically induced barley root tissue in Tris buffer plus DTT gave little LDH activity; inclusion of Na borate and BSA in the extraction buffer greatly increased and stabilized extractable LDH activity (Table I). An extraction buffer containing optimized levels of Na borate (10 mM) and BSA (5 mg/ml) was used for all subsequent work.

Barley roots showed a 20-fold increase in LDH activity in the first 4 to 7 d after N₂ replaced air as the sparging gas, and high LDH levels were maintained during an additional week of hypoxic treatment (Fig. 2). LDH activity was induced throughout the whole root system, although fresh weight basis was highest in the tip region (not shown). Root growth was strongly suppressed during N₂ treatment, but shoot growth was not significantly affected (Fig. 2) and shoots did not differ visibly from those of plants with aerated roots. Roots maintained continuously in aerated conditions showed no change in LDH activity. Mixing experiments in which equal weights of aerobic and anaerobically induced roots were extracted together gave activities which were 89 to 100% of those predicted from extracts of each tissue alone. The addition of aerobic root extract to anaerobic root extract did not destabilize the induced LDH activity during 2 h storage on ice. Anaerobically inducible LDH activity was present in roots of wheat, rye, and maize, although maize roots had considerably higher activity than the other cereals when grown aerobically (Table II).

The physiology of LDH induction was characterized in detail using barley. To determine whether continuous hypoxia was required to maintain high LDH levels, barley roots were sparged with N₂ for various times and returned to aerobic conditions (Fig. 3). In all cases, LDH activity on a fresh weight basis declined with an apparent half-life of about 2 d when aeration was restored. Since root fresh weight increased less than 2-fold in the first 5 d after aeration resumed, the decline in LDH activity in Figure 3 reflects substantial net loss of enzyme activity. Figure 4 demonstrates that LDH activity in barley roots is correlated with the degree of O₂-deficit; thus, the N₂ treatment, which resulted in a measured O₂ level of 0.06%, induced more activity than the other hypoxic treatments (0.4 and 2.6%). Because the roots of these plants were extracted to shoots in air, O₂ levels within at least part of the root system were probably above those measured in solution (2), so that the latter values should be taken as relative rather than absolute. The data of Figure 4 are therefore not directly comparable to those obtained for aleurone layers in a closed system (8), in which LDH activity showed an optimum at 2 to 5% O₂.

Isozyme Profile of LDH. In animals (5) the LDH holoenzyme is a tetramer whose subunits are encoded by two different genes; the products of these genes associate randomly to generate a set of five isozymes. When run on nondenaturing gels, the LDH activity of induced barley roots was resolved into a closely spaced pattern of five bands (Fig. 5). All 5 barley LDH bands migrated between isozymes 2 and 3 of a human LDH standard, indicative of relatively small charge differences among the barley isozymes. The level of LDH activity in aerobically grown barley roots was generally too low to give discernible bands but formed a broad, faintly staining region in the position corresponding to the set of LDH isozymes in induced tissue. It is thus probable that all five isozymes were present in the LDH profile of uninduced roots.

Reproducible quantitative differences were noted in the relative staining intensities of the five barley LDH isozymes as a result of hypoxia and aerobic conditions. The results were consistent with a mechanism in which hypoxia activates a LDH isozyme with a higher O₂ requirement than the baseline enzyme. The level of hypoxia-inducible LDH activity in barley roots was significantly enhanced when N₂ replaced air for a period of 7 d, when it was approximately 2-fold greater than in aerobically grown roots. The LDH activity was maintained in aerated root tissue until the end of the experiment (Fig. 6), indicating that it is a stable enzyme.

Table I. Effect of Buffer Composition on Extractable LDH Activity from Barley Roots

<table>
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<tr>
<th>Concentrations of components:</th>
<th>Tris-HCl (pH 8.5), 100 mM; DTT, 5 mM; BSA, 5 mg/ml</th>
<th>Na borate, 10 mM</th>
<th>Enzyme activity was assayed after the extract had been on ice for various times. Activity is expressed relative to that in Tris-HCl + DTT at 50 min, which was 0.09 IU/g fresh weight.</th>
<th>Relative Enzyme Activity</th>
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<tbody>
<tr>
<td>Addition</td>
<td>Relative Enzyme Activity</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>DTT</td>
<td>BSA</td>
<td>Borate</td>
<td>50 min</td>
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<td>+</td>
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<td>-</td>
<td>-</td>
<td>1.0</td>
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<td>4.7</td>
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<td>+</td>
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<td>6.2</td>
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function of O₂ tension during induction (Fig. 6). When roots were induced by moderate hypoxia (e.g. 1% O₂), isozyme 1 (most anodal) and isozyme 5 stained less intensely than the three central bands. Under more severe hypoxia (0.3% O₂), where total LDH activity was 80% higher, the three most cathodal isozymes (3–5) were the most heavily stained.

Wheat, rye, and maize LDH showed electrophoretic behavior similar to barley (Fig. 6). The wheat LDH profile contained five bands that coincided with the five barley isozymes; maize typically contained two additional cathodal bands. The rye profile was harder to resolve than those of other cereals, although in some gels a pattern similar to barley was seen.

Lactate Accumulation and [¹⁴C]Glucose Experiments. To investigate the in-vivo function of the induced LDH we first measured lactate levels within barley roots during hypoxia (Fig. 2, inset). A small increase (~2-fold) in lactate level occurred within the first day of hypoxia, but there was no further accumulation over the next 3 days, despite the large increase of LDH activity that occurred in the same tissue (Fig. 2). Failure of lactate to accumulate could be due to: (a) absence of lactate synthesis, (b) loss of lactate from the root to the bathing medium, or to the shoot via the transpiration stream, or (c) further metabolism of lactate. To test these possibilities, induced roots were excised and supplied tracer [¹⁴C]glucose at various O₂ tensions. Aseptically grown roots were used because the microorganism populations on roots grown in the standard conditions were potentially high enough (10⁷/g fresh weight) to contribute significantly to the catabolism of the tracer.

In experiments like that of Table III, at head-space O₂ levels below about 1%, ethanol and CO₂ were labeled in a 2:1 ratio, establishing that aerobic metabolism of [¹⁴C]glucose was arrested. Under these conditions, lactate accounted for several percent of the [¹⁴C]glucose catabolized, but ethanol and alanine were always far more heavily labeled; γ-aminobutyrate was also fairly prominently labeled. These four compounds were labeled only slightly in aerobic roots, but together contained about 80% of the total [¹⁴C]-label metabolized to soluble compounds in hypoxic conditions. No other labeled compounds which selectively accumulated in hypoxic roots were seen in autoradiograms, which makes significant metabolism of lactate to other compounds unlikely. The distributions of the main labeled products of hypoxic roots between tissue and medium were dissimilar. The medium contained 96% of the [¹⁴C]ethanol and 77% of the [¹⁴C]lactate, but less than 30% of the [¹⁴C]alanine, 7% of the [¹⁴C]malate, and only 4% of the [¹⁴C]γ-aminobutyrate.

As an approximate basis for converting the proportion of fed [¹⁴C]glucose metabolized to various end products to in vivo glycolytic flux rates, rates of CO₂ production in air were measured for excised aerobic roots and for anaerobically induced roots immediately after transfer to air. Values were 11.3 and 8.5 μmol CO₂/h·g fresh weight, respectively. Assuming that a Pasteur effect is absent, as is the case for rice roots (12), these values predict for hypoxic roots a total glycolytic flux of about 1.65 μmol hexose/h·g.

**DISCUSSION**

Although LDH was detectable in induced roots in the absence of Na borate and BSA, the activity was relatively low and...
unstable. Both BSA and borate presumably afforded protection against phenolics (17) but PVP, another common anti-phenolic, proved ineffective. We also found it essential to include DTT at a minimum of 5 mM to recover LDH activity. It is therefore probable that choice of extraction buffer explains the reported absence of LDH from maize roots (16) and perhaps also the rather low extractable LDH activities found in induced roots of some other species (25).

Barley, wheat, and rye showed a five-banded pattern of hypoxically induced LDH isozymes. This type of pattern is expected in LDH systems where the enzyme is a tetramer and there are two Ldh genes (5). We have shown (11) that barley LDH is tetrameric, and have obtained genetic evidence consistent with a two-gene model (NE Hoffman, AHD Brown, AD Hanson, unpublished data). If we assume a two-gene system in which the gene products associate randomly to form tetramers, when both genes are expressed at the same level the five isozymes will occur in the ratio 1:4:6:4:1. This ratio approximately describes the staining intensities of the five barley isozymes at moderate hypoxia. However, in severe hypoxia when LDH activity is highest, the staining intensity is skewed towards the cathodal isozymes (3–5). One explanation for this is that the gene (Ldh2) whose product forms isozyme 5 is more strongly expressed at very low O2 levels than the gene (Ldh1) which encodes isozyme 1.

That an anaerobically inducible LDH system with multiple isozymes has been conserved in cereal evolution implies that this system has a function related to hypoxia. That LDH activity remains high during prolonged hypoxia but decays upon its relief further implies that this function is exercised throughout hypoxia. What might the function of induced LDH be? The Davies-Roberts hypothesis (4, 21) assigns to LDH the function of cytoplasmic acidification during the first few minutes of hypoxia, and thus deals only with constitutive LDH activity. We therefore hypothesized that induced LDH activity serves to support substantial and continuous lactate glycolysis in roots, there being evidence for this function in barley aleurone layers (8). We also reasoned that lactate might remain in the plant, allowing maintenance of redox balance without the carbon loss inherent in ethanol glycolysis. In tests of these ideas with induced barley roots fed [14C]glucose, lactate was readily detected as an end product of glycolysis, but was fairly minor and was lost to the medium.

Thus, the flux of 14C to lactate in hypoxic, induced roots (about 3%) was far smaller than that to ethanol (46%) and alanine (47%). This is quite different from barley aleurone tissue, where lactate accounts for up to 35% of the total flux and alanine for less than 10% (8). Taking the total glycolytic flux in induced roots as 1.65 μmol hexose/h·g, the rate of lactate formation

![Figure 5](image-url) - The full complement of barley LDH isozymes. On the left are marked the running positions of human LDH-3 and LDH-2. On the right is an interpretation of the barley LDH isozymes which assumes that the holoenzyme is a tetramer made up of subunits (LDH1, LDH2) encoded by two different genes.

![Figure 6](image-url) - LDH isozyme profiles of four cereals. Roots were sparged with N2/air mixtures containing 0.3% or 1% O2 for 5 d before extraction.
Table III. Effect of Hypoxia on Metabolism of [U-14C]Glucose by Induced Barley Roots

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<tr>
<th></th>
<th>Aerobic</th>
<th>Hypoxic</th>
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<td>% of metabolized 14C recovered as:</td>
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<tr>
<td>CO2</td>
<td>34.3</td>
<td>12.4</td>
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<tr>
<td>Ethanol-insoluble</td>
<td>33.4</td>
<td>4.3</td>
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</table>

Our results establish that cereal roots possess a set of LDH isozymes which appear during hypoxia, and whose in vitro catalytic potential is large. However, the functional significance of the induced enzymes is not clear. Thus, lactate glycolysis was present in induced roots but was a minor carbon flux compared to ethanol glycolysis and alanine synthesis. While it is plausible that lactate glycolysis was in effect suppressed by the high N status of our experimental plants, our data encourage speculation that LDH has some as yet unrecognized function. In this context, it is interesting that Williams et al. (26) recently identified a DNA helix destabilizing protein from rat liver as LDH-5, and suggested that LDH-5 might play a role in transcription as well as in glycolysis.

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