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Control of Lactate Dehydrogenase, Lactate Glycolysis, and α-Amylase by O_2 Deficit in Barley Aleurone Layers[1]

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ABSTRACT

After 4 days in an atmosphere of N_2, aleurone layers of barley (*Hordeum vulgare* L. cv Himalaya) remained viable as judged by their ability to produce near normal amounts of α-amylases when incubated with gibberellic acid (GA_3) in air. However, layers did not produce α-amylase when GA_3 was supplied under N_2, apparently because α-amylase mRNA failed to accumulate.

When an 8-hour pulse of [U-^{14}C]glucose was supplied under N_2 to freshly prepared aleurone layers, both [^{14}C]lactate and [^{14}C]ethanol accumulated; the [^{14}C]lactate/[^{14}C]ethanol ratio was about 0.3. Prior incubation of layers for 1 day under N_2 changed this ratio to about 0.8, indicating an increase in the relative importance of the lactate branch of glycolysis.

L(+)Lactate dehydrogenase (LDH) activity was low in freshly prepared aleurone layers and increased 10-fold during 2 days under N_2, whereas alcohol dehydrogenase activity (ADH) was high initially and rose by 60%. The responses of LDH and ADH activities to O_2 tension were dissimilar; when layers were incubated in various O_2/N_2 mixtures, LDH activity peaked at 2 to 5% O_2 whereas ADH activity was highest at 0% O_2. The LDH activity was resolved into several enzymically active bands by native polyacrylamide gel electrophoresis.

We conclude that barley aleurone layers are highly adapted to O_2 deficiency, that they possess an inducible LDH system as well as an ADH system, and we infer that the LDH and ADH systems are independently regulated.

Compared to rice (*Oryza sativa*) and wild rice (*Zizania aquatica*), cereals like barley and maize are intolerant of flooded and waterlogged soils. A primary lesion in flood-intolerant plants is failure of glycolytic pathways in root tissues to cope with low O_2 tension, especially when this is chronic (4, 7). This failure could occur because genetic information for glycolytic pathways competent at low O_2 tension is absent from flood-intolerant cereals or because (although present) such information remains more or less silent in mature root systems. Evidence indicating that cereal seed and seedling tissues normally have very high constitutive levels of ADH,[3] and are highly tolerant of O_2 deprivation, favors the second possibility. Thus, imbibed seeds or germinating seedlings of wild-type barley and maize survive severe O_2 deficiency for several days (12, 14, 26, 27), whereas null mutants at the principle alcohol dehydrogenase locus (*Adh1* in both species) do not (14, 27).

There are both experimental and theoretical considerations which indicate that glycolytic pathways other than that leading to ethanol may operate in anaerobic seed and seedling tissues. Lactate has been reported to occur in germinating maize and barley (6, 22) as well as in legume seeds (21). Comparative biochemistry of both animals and plants tolerant of anoxia suggests that a common stratagem for surviving without O_2 is to diversify glycolytic end products (4, 16). Besides lactate and ethanol, various organic acids, amino acids, and opines have been proposed or found to accumulate during anaerobic glycolysis in plants and/or animals (4, 7, 17).

In the work reported in this and the following paper (13), we have used aleurone layers of barley to answer a series of questions, prompted by the considerations outlined above, relating to survival, metabolism, and gene expression in barley seed during O_2 deficit. This paper poses the following questions. (a) How well do the tissues survive and function during anoxia? (b) What are the principal glycolytic pathways operating during anoxia? (c) How do activities of enzymes catalyzing the principle glycolytic pathways—LDH and ADH—respond to O_2 deficit? Aleurone tissues were used in preference to embryos because of their cellular uniformity and their nonmeristematic, nongrowing nature, which eliminate many possible confounding effects.

MATERIALS AND METHODS

Preparation and Incubation of Aleurone Layers. Aleurone layers were prepared in air from barley (Hordeum vulgare L. cv Himalaya; 1979 harvest; University of Washington, Pullman, WA) essentially according to Chrispeels and Varner (5). Batches of 10 layers were incubated under sterile conditions in loosely plugged 25-ml Erlenmeyer flasks containing 2 ml of incubation medium (10 mM $CaCl_2$ plus 10 μg/ml chloramphenicol) unless otherwise indicated. For routine incubations under N_2, flasks were transferred to a 2-L desiccator which was purged for 30 min with high-purity N_2; the desiccator was then placed on a reciprocal shaker (60 cycles/min) at 24 ± 2°C. For incubations lasting more than 1 d, the desiccator was repurged after 4 h on the same day and daily thereafter with N_2 for 15 min. Controlled atmosphere experiments (Fig. 4) were conducted using sealed 0.9-L jars containing two or three flasks. The jars were first flushed with N_2 for 1 to 1.5 h. O_2 was then injected, and N_2 simultaneously withdrawn, to give the desired gas mixtures. Control flasks were incubated in air, not enclosed in jars. The O_2 levels in the jars were measured by GLC (29) 3 h after O_2 injection and just before the end of the 2-d incubation; the two measurements differed by <10% and were averaged.

GA_3 Induction of α-Amylases. Batches of 10 layers were placed in 2 ml incubation medium containing 1 μM GA_3 and incubated

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[3] Abbreviations: ADH, alcohol dehydrogenase; LDH, lactate dehydrogenase, PDC, pyruvate decarboxylase; ANPs, anaerobic polypeptides; CPI, Commonwealth Plant Introduction Number.

in air on a reciprocal shaker for 24 h at 24 ± 2°C. Each batch of layers was then ground in its own incubation medium in a mortar and pestle with the aid of some acid-washed sand. After clarifying the extract by centrifugation, α-amylase activity was measured using the assay of Chrispeels and Varner (5). Total α-amylase was fractionated into A and B groups of α-amylase by CMC-cellulose column chromatography using a modification of the procedure of MacGregor *et al.* (23). The buffer was 0.02 M succinate (pH 4.3) containing 10 mM $CaCl_2$ and the column was eluted with a linear 0 to 1.2 M NaCl gradient at room temperature. The identity of the two amylase peaks as α-amylase A and B was established by agar gel electrophoresis (19).

^{35}S Labeling and Separation of Polypeptides. Four batches of 10 aleurone layers were incubated in 2 ml of 10 mM $CaCl_2$ in air or N_2 for 6 h, at which time GA_3 (final concentration, 1 μM) was added to two batches in each atmosphere. After incubation for a further 17 h, layers were rinsed thoroughly in distilled H_2O, maintaining anaerobic conditions for the N_2-treated samples. One ml of a solution containing 10 mM $CaCl_2$ (with or without GA_3) and 20 μCi L-[^{35}S]methionine (>800 Ci/mmol; Amersham) was added to each flask and incubation in air or N_2 continued for another 6 h. Labeled polypeptides were extracted by grinding batches of layers in 2 ml of 0.15 M Tris-HCl buffer (pH 8.0) plus 10 mM DTT. After clarifying the extract by centrifugation, proteins were precipitated by adding 5 volumes of cold acetone and standing at −15°C for about 1 h. The precipitated proteins were dissolved in SDS-PAGE sample buffer (28) and separated on gradient gels (12.5–20% polyacrylamide) according to Spencer *et al.* (28). Radioactivity was detected by fluorography (2).

RNA Isolation and *In Vitro* mRNA Translation. RNA was isolated, and then translated in a cell-free system derived from wheat germ in the presence of [^{35}S]methionine as described previously (15). Newly synthesized polypeptides were fractionated by SDS-PAGE and detected by fluorography as described above.

[^{14}C]Glucose Experiments and Recovery of Labeled Compounds. Sterile conditions were maintained throughout. Batches of 10 layers were first rinsed thoroughly in distilled H_2O, and then transferred to 25-ml flasks with 1.0 ml of water containing 20 μg chloramphenicol and 0.25 μCi D-[U-^{14}C]glucose (296 mCi/mmol; Amersham). Flasks were sealed with serum caps, flushed with filtered N_2 for 5 min, and incubated for 8 h in a shaking water bath at 24 ± 2°C. The incubations were stopped by adding 5.0 ml of absolute ethanol plus 1 mg unlabeled carrier glucose, and holding at −15°C for at least 1 h. Flasks were then connected to a small condenser, and heated in a boiling water bath; about 3 ml of distillate were collected. The distillate was bubbled with N_2 to drive off remaining $^{14}CO_2$, and its specific gravity (and hence ethanol content) determined. A sample of distillate was taken for scintillation counting; total [^{14}C]ethanol production was calculated from the ^{14}C and ethanol contents of the distillate.

Liquor remaining in the incubation flasks was drawn off and the tissue re-extracted with 5 ml of 80% (v/v) ethanol at room temperature. The ethanolic extracts were pooled, reduced to dryness *in vacuo* at 30°C, redissolved in 2.0 ml H_2O, and fractionated as detailed below.

Fractionation of Nonvolatile [^{14}C]Products. The ethanol extract was separated into cationic, anionic, and neutral fractions using BioRad AG-50 W × 8 (H^+) and AG-1 × 8 (formate) resins, essentially as described by Canvin and Beevers (3). Small samples of fractions were taken for scintillation counting, and the remainder reduced to dryness *in vacuo* at 30°C. The anionic fraction, containing any [^{14}C]lactate present, was analyzed further by TLC on precoated cellulose plates (0.1 mm; Merck), developed for 2 h with *n*-butanol:acetone:diethylamine:H_2O (30:30:6:15, v/v, solvent A), or for 4 to 5 h with *n*-butanol:glacial acetic acid:H_2O (60:15:25, v/v, solvent B). Organic acid standards were detected with an indicator spray (0.04% bromophenol blue in 96% ethanol); radioactive zones were detected on TLC plates by autoradiography. [^{14}C]Lactate was quantified using TLC plates developed with solvent A; the labeled lactate zone was scraped from plates and eluted in 1 ml of H_2O before scintillation counting.

Amino acids present in the cationic fraction were also analyzed using both the TLC systems above. The neutral fraction was subjected to descending paper chromatography on Whatman No. 1 paper developed overnight in *n*-propanol:ethyl acetate:H_2O (7:1:2, v/v). Radioactive zones were detected by autoradiography, and identified by co-chromatography with authentic standards.

Derivatization of [^{14}C]Ethanol and [^{14}C]Lactate. Representative samples of [^{14}C]ethanol and [^{14}C]lactate, isolated as above, were converted to solid phenylurethane and *p*-bromophenacyl ester derivatives repectively. For [^{14}C]ethanol, 1-ml aliquots of distillate were treated with 2 ml of phenylisocynate; the crude urethane product was dissolved in methylene chloride and applied to a 2- × 20-cm Kieselgel column. The column was eluted with petroleum ether:methylene chloride (1:1, v/v); the fractions containing urethane were identified by TLC and pooled, and the solvent removed by rotary evaporation. The product was then recrystallized from petroleum ether. For [^{14}C]lactate, dried eluates from the lactate zones of TLC plates developed with solvent A were dissolved in 0.3 ml H_2O and mixed with 5.0 ml of 0.5 M sodium-DL-lactate (pH 4.5). After refluxing with 0.77 g *p*-bromophenacylbromide in ethanol for 1 h, the ester was precipitated from the reaction mix by adding about 3 volumes of water and cooling. The ester was purified on a Kieselgel column as above, except that the eluting solvent was methylene chloride, and then recrystallized from 25% ethanol. The identities of the ethanol and lactate derivatives were confirmed by NMR, and weighed samples taken for scintillation counting to determine their specific radioactivity.

Extraction and Assay of LDH and ADH. Batches of 10 layers were ground with sand in an ice-cold mortar and pestle in 2.0 ml of 0.15 M Tris-HCl (pH 8.0) containing 10 mM DTT. The brei was clarified by centrifugation and aliquots of the supernatant taken for spectrophotometric assays of LDH and ADH.

LDH was routinely assayed using the approach of Davies *et al.* (9), *i.e.* in the pyruvate→lactate direction, monitoring pyruvate-dependent NADH oxidation at 340 nm at alkaline pH in the presence of a pyrazole ADH inhibitor. In place of pyrazole itself, used by Davies *et al.*, the derivative 4-bromopyrazole was substituted because it has a K_i about 7-fold lower (25). At a concentration of 10 mM in the reaction mix, 4-bromopyrazole suppressed ADH activity completely. The assay mix (final volume 3.0 ml) contained 1 ml of 0.5 M Tris-HCl (pH 8.0), 400 μg NADH, 30 μmol 4-bromopyrazole, 30 μmol sodium pyruvate, and 0.4 ml enzyme extract. The 4-bromopyrazole was added to the mix in 10 μl of acetone before starting the reaction with pyruvate. In some experiments, LDH was also assayed in the lactate→pyruvate direction. The reaction mix (final volume 3.0 ml) contained 1 ml of 0.5 M Tris-HCl (pH 9.0), 100 μmol lithium lactate (L[+] or D[−]), 3 μmol NAD, 30 μmol 4-bromopyrazole, and 0.4 or 0.5 ml enzyme extract. LDH activities are reported in IU (μmol/min).

ADH activity was assayed in the ethanol→acetaldehyde direction in a 3-ml reaction mix containing 1 ml of 0.5 M Tris-HCl (pH 9.0), 3 μmol NAD, 100 μmol ethanol, and 0.1 ml enzyme extract. ADH activities are given in IU.

Polyacrylamide Gel Electrophoresis of LDH. Native slab PAGE was performed as follows. Layers were ground as above in 75 mM Tris·HCl (pH 8.5) plus 10 mM DTT (10 layers/ml), and the extract clarified by centrifugation. Each gel track typically

contained 85 μl of supernatant plus 15 μl of 90% glycerol containing 0.01% bromophenol blue. The running gel contained (w/v) 7.5% acrylamide-0.1% *N,N'*-methylene bisacrylamide (bis) and 0.38 M Tris·HCl (pH 8.5), polymerized with 0.08% (v/v) *N,N,N',N'*-tetramethylene ethylene diamine (TEMED), and 0.036% ammonium persulfate. The stacking gel contained 2.5% acrylamide-0.63% bis, 75 mM Tris·HCl (pH 8.5), 20% (w/v) sucrose, 0.06% TEMED, and 0.0005% riboflavin, and was photopolymerized for about 1.5 h, 15 cm from a fluorescent tube. Gels were run in the cold room with 25 mM Tris-190 mM glycine buffer (pH 8.5) in both electrode tanks, at 10 mamps until the dye front entered the running gel, and then at 15 mamps overnight. The dye front migrated about 15 cm through the running gel. Gels were stained in a solution containing 50 ml of 0.15 M Tris·HCl (pH 8.0), 1.0 g lithium L(+)lactate, 100 mg NAD, 20 mg $MgCl_2$, 10 mg Nitro Blue Tetrazolium, 2.5 mg of phenazine methosulfate, and 150 μmol of 4-bromopyrazole (added to inhibit trace staining of ADH bands). No bands were stained when lactate was omitted.

RESULTS

Survival of Aleurone Tissue during Incubation under N_2. As a convenient measure of tissue function, we assayed the total α-amylase activity produced in the presence of 1 μM GA_3 during a 24-h incubation period in air. By this measure, layers incubated under N_2 for 4 d suffered only about 40% loss of function, which was less than the loss in layers incubated for 4 d in air (Fig. 1). Fractionation of total α-amylase activity showed that both A- and B-group α-amylases were produced after 4 d N_2 treatment, with depressed B-group activity accounting for most of the difference from control layers (Fig. 1, inset). Separate genes specify the α-amylases of groups A and B, and the timing of expression of these genes in response to GA_3 is different, with A somewhat preceding B (18). Thus, the observed lowering of B-group activity would be explained were N_2 treatment merely to slow the response to GA_3. Consistent with this, intact grains imbibed under N_2 for 2 to 4 d subsequently germinated (>90%) normally, but more slowly than controls.

Although aleurone layers responded well to GA_3 in air following prolonged pre-incubation under N_2, they produced little or no α-amylase activity over periods up to 2 d when GA_3 was supplied under N_2. Since GA_3 causes radical changes, both increasing and decreasing the synthesis of specific polypeptides in aerobic aleurone layers (15), we were prompted to determine if N_2 prevented all of the effects of GA_3, or only the appearance of enzymically active α-amylase. We therefore directly compared the profiles of labeled polypeptides synthesized in air and N_2, minus and plus GA_3 (Fig. 2). In the absence of GA_3, the pattern of polypeptides synthesized under N_2 (anaerobic polypeptides, ANPs) was considerably simpler than the aerobic profile, as in maize roots (26). Although in air, GA_3 effected a major redirection of protein synthesis, stimulating α-amylase synthesis and suppressing synthesis of many other polypeptides, it perturbed the ANP profile only slightly and promoted only a trace of α-amylase synthesis.

To obtain an indication of the mechanism by which anoxia prevents GA_3-promoted α-amylase synthesis, we examined the effect of N_2 on translatable mRNA levels. RNA was extracted from aleurone layers treated as described above for Figure 2, and mRNA assayed by *in vitro* translation. Very little α-amylase mRNA was detected and other effects on the translation product profile were minor when GA_3 was added to N_2-treated aleurone layers, whereas, as described previously (15), there was a greatly increased level of translatable α-amylase mRNA in aleurone treated with GA_3 in air (data not shown). Thus, the effects of GA_3 on mRNA levels in N_2 mirror those on protein synthesis indicating that anaerobiosis prevents α-amylase synthesis by preventing the accumulation of its mRNA.

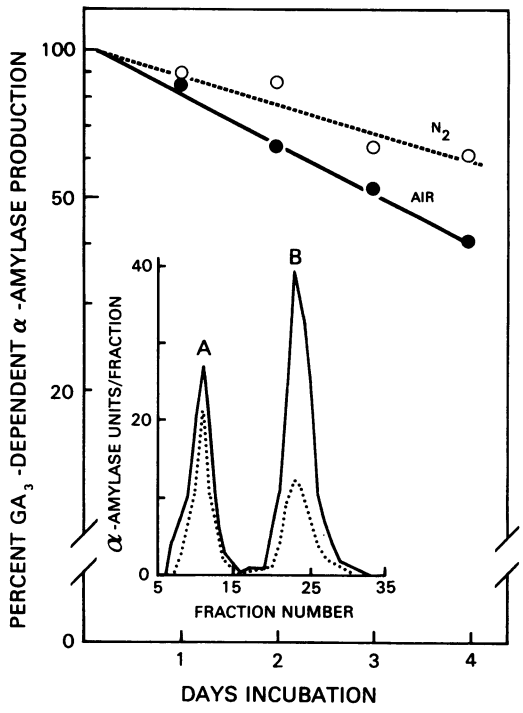

FIG. 1. Retention of the GA_3 response by aleurone layers after incubation without GA_3 for various times in N_2 or air. After the incubation treatment, batches of 10 layers were transferred to fresh medium containing 1 μM GA_3 and incubated in air for 24 h. The total α-amylase present in medium plus layers was determined. Results from six experiments were pooled; N_2 data points are the average of five to nine batches of layers, air data points are the average of three to seven batches. Results are expressed as a percentage of the α-amylase produced in each experiment by control layers which were not incubated in air or N_2 before GA_3 addition; α-amylase production by these controls averaged 55 units per 10 layers (range 37–79). In the absence of GA_3, α-amylase production was always very low (about 1–3 units per 10 layers). Inset shows CMC-cellulose fractionation into A and B groups of the total α-amylase produced in 24 h by 50 control, nonincubated layers (——) and 50 layers incubated for 4 d under N_2 (·····).

Glycolytic End Products of Glucose. Pyruvate (or phosphoenolpyruvate) is derived from hexose, and is an intermediate in all known glycolytic pathways. We therefore reasoned that (a) the glycolytic pathways present in aleurone layers could be identified from the types of labeled end products accumulated during incubation under N_2 with [U-^{14}C]glucose, and (b) the proportions of different labeled end products would closely reflect the relative importance of their corresponding pathways *in vivo*, provided that all pathways diverge from the same pool of pyruvate.

In all experiments, most of the [^{14}C]glucose catabolized during 8 h under N_2 was recovered in the ethanol distillate fraction, and in an anionic product that co-migrated with authentic lactate in 2 TLC systems. Derivatization of typical distilled material and eluates from the lactate TLC zone confirmed the presence and radiochemical purity of [^{14}C]ethanol and [^{14}C]lactate (Fig. 3). The ^{14}C content of the distilled fraction was therefore taken to be [^{14}C]ethanol, and that of the lactate TLC zone to be [^{14}C]lactate, for the measurements of Table I.

Freshly prepared aleurone layers catabolized no more than 15% of the supplied [^{14}C]glucose, whereas layers previously incubated in air or N_2 catabolized about 90% and 40 to 70%, respectively (Table I). These differences are at least partly explained by a marked reduction of the levels of free sugars (glucose,

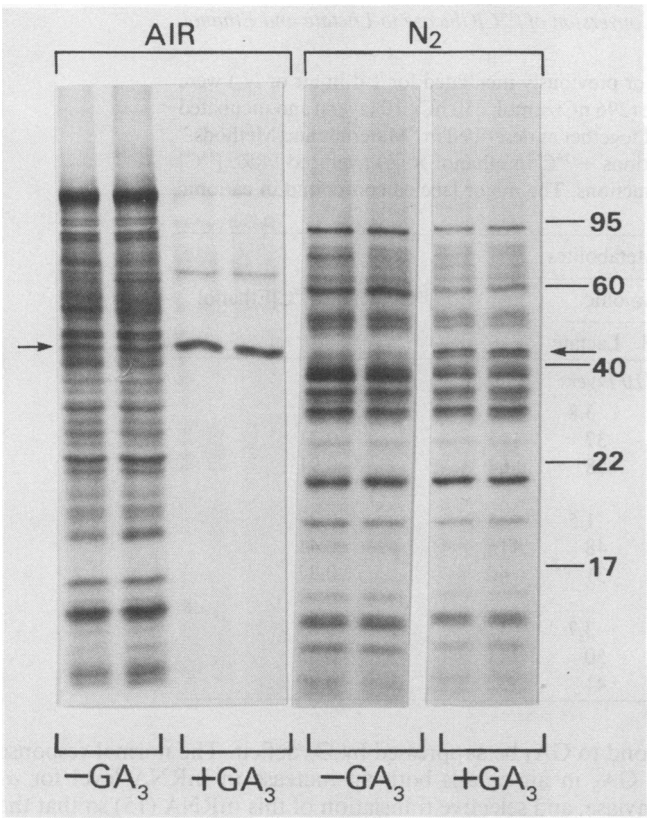

FIG. 2. Fluorographs of SDS-PAGE separations of [^{35}S]polypeptides extracted from aleurone layers incubated for 29 h in air or N_2, with 1 μM GA_3 present during the last 23 h, or absent throughout. Layers were supplied with [^{35}S]methionine (20 μCi/10 layers) for the final 6 h of incubation. Each track contains the protein equivalent of one aleurone layer. Tracks for samples incubated in N_2 were exposed to x-ray film 8 times longer than tracks for air samples. Arrows mark the α-amylase zone. The numbers (mol wt $\times 10^{-3}$) indicate the positions of mol wt markers and they apply to both air and N_2 treatments.

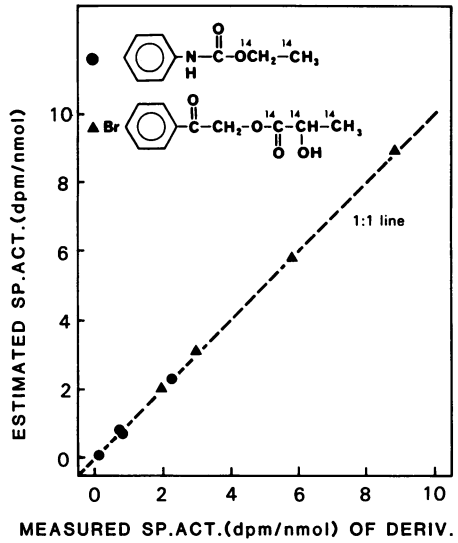

FIG. 3. Confirmation of the identity and radiochemical purity of [^{14}C]ethanol and [^{14}C]lactate samples prepared by the standard fractionation procedure (see "Materials and Methods"). For both [^{14}C]ethanol and [^{14}C]lactate, known amounts of label and unlabeled authentic carrier were mixed, and the indicated derivative prepared, purified, and verified by nuclear magnetic resonance. The abscissa is the specific radioactivity of the purified derivative. The ordinate is the specific activity of ethanol or lactate estimated by assuming all label in the distilled material or the lactate TLC zone to be in the form of [^{14}C]ethanol or [^{14}C]lactate, respectively. The presence of other [^{14}C] products would cause the points to fall above the 1:1 line.

fructose, sucrose) in preincubated layers that were detected by paper chromatography (data not shown), and do not affect interpretation of the [^{14}C]lactate/[^{14}C]ethanol ratios. This ratio was 0.2 to 0.3 in freshly prepared layers, increased to about 0.4 in layers previously incubated in air, but increased to 0.8 to 0.9 in layers previously incubated in N_2.

In molar terms, a [^{14}C]lactate/[^{14}C]ethanol ratio of 0.8 corresponds to a 35:65 split between the lactate and ethanol branches of glycolysis, a ratio of 0.2 to a 12:88 split.

Time Courses for LDH and ADH Activities under N_2. Because *in vivo* labeling experiments implicated an LDH in the response to anaerobiosis, such an activity was sought in crude extracts, along with ADH. Figure 4 presents time courses for the activities of these two enzymes under N_2. Both enzyme activities exhibited anaerobic induction, with LDH increasing about 10-fold, and ADH rising by about 60% over 2 d. LDH activity also increased about 2-fold during incubation in air, although ADH activity did not.

The LDH present in crude extracts of N_2-treated layers was about 15-fold more active in the pyruvate→lactate direction than in the opposite direction under the standard assay conditions. The K_m for pyruvate was 2.5 mM (pH 8.0, 0.11 mM NADH). The K_m for NADH was 20 μM (pH 8.0, 10 mM pyruvate). Assayed in the pyruvate→lactate direction, the pH optimum was 7.0. No activity with D(−)lactate was detected. These characteristics are similar to those reported for LDH from various plant sources (*e.g.* 1, 8, 21). It is improbable that the LDH activity assayed (or the [^{14}C]lactate accumulation observed) was due to microbial contamination, since contamination checks of media and aleurone layers on potato dextrose agar and nutrient broth agar plates proved negative, and nonsterile samples with obvious microbial growth showed lower LDH activity than normal.

Effect of O_2 Tension on LDH and ADH. Although ADH activity increased as the O_2 tension was decreased from 21% to 0%, LDH activity increased to a maximum in the 2 to 5% O_2 range and declined at 0% O_2 (Fig. 5). Inasmuch as total ADH activity increased as O_2 tension fell from 2% to 0%, and the anaerobic induction of ADH isozymes associated with the *Adh 2* and *Adh 3* genes is known to be strongest below 2% O_2 (12, 13), it is unlikely that the observed drop in LDH activity at 0% O_2 is due to a general loss in capacity for protein synthesis or to a general increase in protein breakdown.

When extracts of aleurone layers induced at various O_2 tensions were separated by PAGE using a pH-continuous buffer system and stained for LDH activity, several enzymically active bands were detected (Fig. 5, inset). These multiple forms of LDH were apparently induced in parallel as a function of O_2 tension. Following induction for 2 d at 2% O_2, very similar multiple LDH bands were given by aleurone layer extracts of four other genotypes that were tested: three *H. vulgare* cultivars unrelated to cv Himalaya (Betzes, Clipper, CPI 77169) and one *H. spontaneum* line (CPI 77144) (results not shown).

DISCUSSION

Adaptation of Aleurone Layers to O_2 Deficit. Because our results confirm that mature aleurone cells of barley are highly resistant to O_2 deficits, the barley genome must specify glycolytic machinery capable of functioning in these conditions. Aleurone layers express a constitutive capacity for ethanol glycolysis and an inducible capacity for lactate glycolysis. The latter is of particular interest since it appears to be a novel finding. We

Table I. *Effect of Previous Incubation in Air or N_2 on the Conversion of [^{14}C]Glucose to Lactate and Ethanol under N_2*

Duplicate batches of 10 aleurone layers (freshly stripped or previously incubated for 1 d in air or N_2) were transferred to fresh medium containing tracer [U-^{14}C]glucose (296 nCi/nmol; 250 nCi/10 layers) and incubated for 8 h under N_2. Incubation media and layers were extracted together as described in "Materials and Methods." Overall ^{14}C recovery (^{14}C in cationic, anionic, neutral fractions + ^{14}C in ethanol × ½) averaged 98%. [^{14}C] Glucose was the only labeled compound found in neutral fractions. The major labeled compound in cationic fractions co-chromatographed with alanine.

Expt	Preincubation	[^{14}C]Glucose Catabolized	[^{14}C]Metabolites				[^{14}C]Lactate/[^{14}C]Ethanol
			Cationic	Anionic		Ethanol	
				Total	Lactate		
		%	*nCi/10 layers*				
1	None	15	4.7	6.3	3.8	18	0.21
	Air	88	3.4	43	37	115	0.31
	N_2	68	12	62	58	64	0.91
2	None	5.4	3.0	2.1	1.5	5.6	0.27
	Air	92	5.1	52	48	116	0.41
	N_2	43	14	33	31	40	0.77
3	None	10	4.5	4.7	3.7	11	0.32
	Air	90	4.4	54	50	111	0.45
	N_2	54	12	45	41	52	0.80

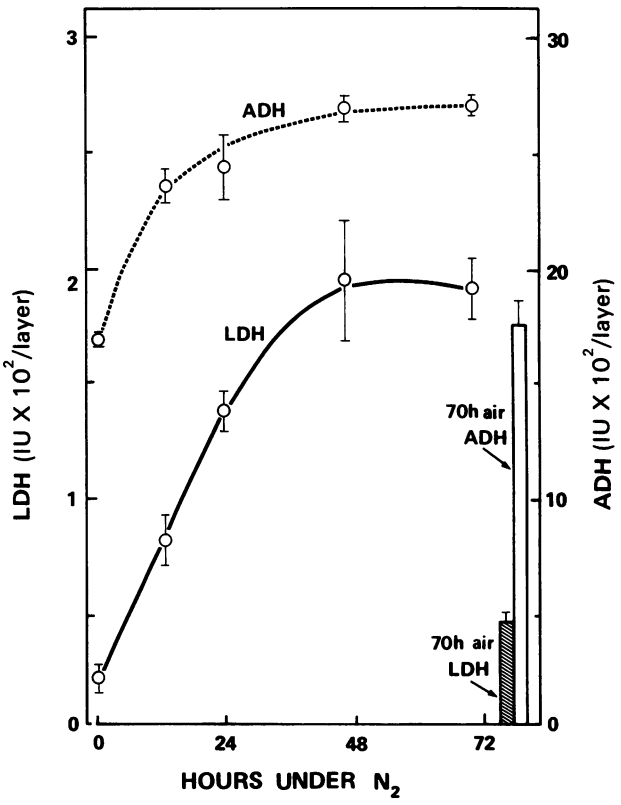

FIG. 4. Time courses for LDH and ADH activities extracted from aleurone layers incubated under N_2 for various times. Bars show activities in control layers incubated for 70 h in air. Data are means ± SE for triplicate samples, each of 10 layers. The experiment was repeated, with similar results.

speculate that inducible lactate glycolysis contributes much to the remarkable tolerance of aleurone layers to O_2 deficit.

Perhaps the induction and maintenance of the enzymes of lactate and ethanol glycolysis requires that the capacity to respond to GA_3 be suppressed by O_2 deficit. The normal response to GA_3 in air entails both an increase in mRNA level for α-amylase, and selective translation of this mRNA (15) so that the observed repression of expression of α-amylase genes during anoxia could be due to control of mRNA level or of its translation. The *in vitro* mRNA translation experiment indicates that failure of α-amylase mRNA to accumulate is probably the major controlling factor in the inhibition of α-amylase synthesis although there is evidence for both types of controls from work on the ANPs of maize (11, 26). In maize roots, anaerobic conditions not only increase the levels of mRNA for ADH and a small number of other ANPs, but also suppress the translation of mRNA for many polypeptides produced in aerobic conditions. Prolonged anaerobiosis also reduces the levels of translatable message for the aerobic polypeptides. It is certain that the ANPs of barley, like those of maize, include ADH (13), and probable that they include other enzymes of glycolysis such as LDH (see below), so that ANP induction in aleurone tissue can be seen as an adaptive phenomenon. If this is so, then competition between the anaerobic induction of ANPs and GA_3 induction of α-amylase could be maladaptive, because the redirection of polypeptide synthesis by GA_3 in air greatly reduces the production of polypeptides other than α-amylase. Also, high α-amylase production and rapid starch hydrolysis is unlikely to be needed when O_2 availability limits growth of the embryo.

Induction of Lactate Glycolysis and LDH Activity. That ^{14}C labeling experiments showed lactate to gain prominence as an end product of glycolysis after prolonged hypoxia was initially surprising, inasmuch as the opposite has been found to be the case in systems where the titers of glycolytic enzymes do not change (7, 8). However, the anaerobically induced LDH activity detected in aleurone tissue can satisfactorily explain the [^{14}C] lactate data. It is worthwhile to note that in the case of fresh aleurone layers and layers previously incubated in air, considerable LDH induction probably occurred during the 8-h anaerobic incubation with [^{14}C]glucose since a 4-fold increase in LDH activity had occurred after 12 h under N_2 (Fig. 4). The data of Table I may therefore overestimate the relative importance of lactate glycolysis in layers exposed for the first time to anoxia.

Although LDH has been isolated from several plant sources,

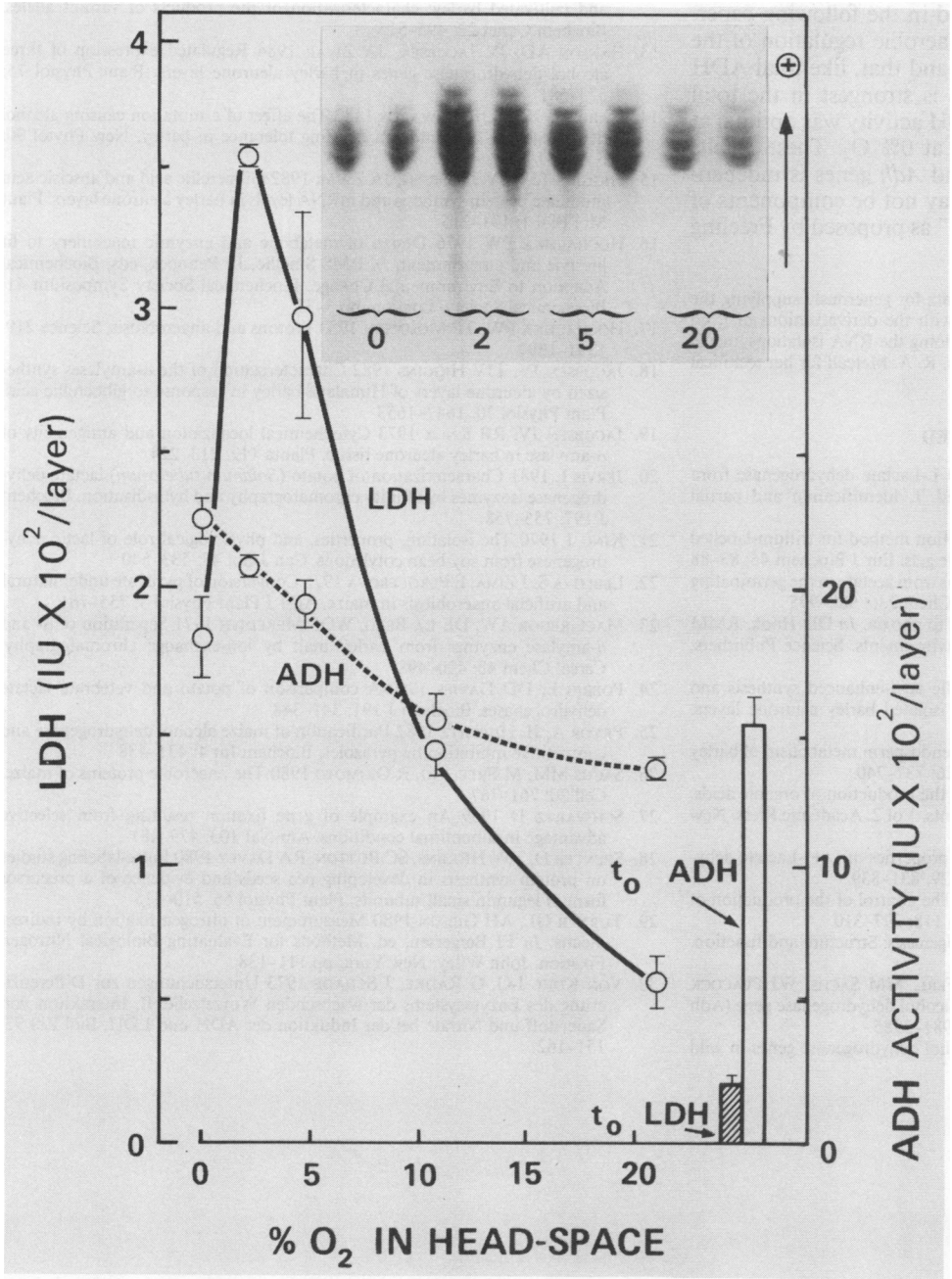

FIG. 5. LDH and ADH activities extracted from aleurone layers incubated for 2 d in various O_2/N_2 mixtures. Bars show the activities present before incubation. Data are means ± SE for triplicate samples, each of 10 layers. The experiment was repeated three times, with similar results. Inset (from a separate experiment) shows multiple forms of LDH resolved by PAGE from extracts of layers incubated for 2 d in 21%, 5%, 2%, and 0% O_2. There are two tracks for each O_2 level; each track contained a volume of extract equivalent to 0.85 layers.

there is very little work reported on LDH induction during anaerobiosis. Nor is there extensive literature for plants on the isozyme composition of LDH and its genetic control (Refs. 1, 20, 24 and citations therein). With respect to anaerobic induction, von Kohl *et al.* (30) reported 2- to 3-fold stimulations of LDH in maize seedling roots, but there were similar stimulations of ADH activity and there is no indication that these authors took the precaution of inhibiting ADH when conducting LDH assays. It is therefore unclear how much, if any, of the reported LDH activity was real. The dearth of information on protein structure and genetic control of plant LDH systems contrasts sharply with the situation for the LDH systems of vertebrates, which are well studied physiologically, biochemically, and genetically (see Ref. 10 for review). Vertebrate LDHs are tetrameric enzymes with monomers having a mol wt of about 35,000; monomer products of different *Ldh* genes can combine to generate a range of isozymes; in the common case where there are two *Ldh* genes, there are five isozymes. It is intriguing that recent biochemical work on potato LDH isozymes indicates that these enzymes closely resemble those of vertebrates (20, 24). The multiple banding pattern for barley LDH that we report is not unlike patterns generated by potato and vertebrate LDHs in various gel systems, suggesting that the barley LDH forms could be tetramers with monomeric mol wt of 35 to 50,000. Although anaerobic induction of ADH in aleurone tissue has been shown to involve *de novo* ADH synthesis (13), our results do not allow us to make similar conclusions about LDH. However, the ANPs of aleurone tissues certainly include polypeptides with mol wt 35 to 50,000, the expected mol wt of LDH monomers.

Relationship between LDH and ADH Induction. Although there is a high level of ADH in mature aleurone layers which have not been experimentally deprived of O_2, and an associated constitutive capacity for ethanol glycolysis, there is nonetheless an anaerobically inducible component of ADH activity. This

anaerobic induction of ADH is covered in the following paper, but it is pertinent to note here that anaerobic regulation of the expression of 3 *Adh* genes is involved, and that, like total ADH activity, the expression of these genes is strongest in the total absence of O_2. In contrast to ADH, LDH activity was optimal at low O_2 levels, and declined markedly at 0% O_2. These results indicate that expression of the *Ldh* and *Adh* genes is independently regulated, so that these genes may not be components of a "co-adapted (anaerobic) gene battery," as proposed by Freeling and co-workers for maize ANPs (26).

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