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Regulated Expression of Three Alcohol Dehydrogenase Genes in Barley Aleurone Layers[1]

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ABSTRACT

Three genes specify alcohol dehydrogenase (EC 1.1.1.1.; ADH) enzymes in barley (*Hordeum vulgare* L.) (*Adh 1*, *Adh 2*, and *Adh 3*). Their polypeptide products (ADH 1, ADH 2, ADH 3) dimerize to give a total of six ADH isozymes which can be resolved by native gel electrophoresis and stained for enzyme activity.

Under fully aerobic conditions, aleurone layers of cv Himalaya had a high titer of a single isozyme, the homodimer containing ADH 1 monomers. This isozyme was accumulated by the aleurone tissue during the later part of seed development, and survived seed drying and rehydration. The five other possible ADH isozymes were induced by O_2 deficit. The staining of these five isozymes on electrophoretic gels increased progressively in intensity as O_2 levels were reduced below 5%, and were most intense at 0% O_2.

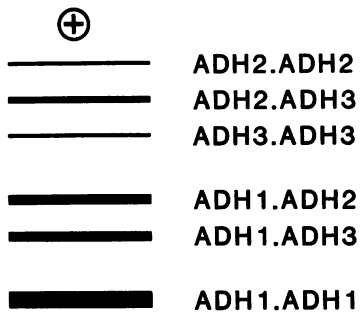
***In vivo* ^{35}S labeling and specific immunoprecipitation of ADH peptides, followed by isoelectric focusing of the ADH peptides in the presence of 8 molar urea (urea-IEF) demonstrated the following. (a) Aleurone layers incubated in air synthesized ADH 1 and a trace of ADH 2; immature layers from developing seeds behaved similarly. (b) At 5% O_2, synthesis of ADH 2 increased and ADH 3 appeared. (c) At 2% and 0% O_2, the synthesis of all three ADH peptides increased markedly.**

Cell-free translation of RNA isolated from aleurone layers, followed by immunoprecipitation and urea-IEF of *in vitro* synthesized ADH peptides, showed that levels of mRNA for all three ADH peptides rose sharply during 1 day of O_2 deprivation. Northern hybridizations with a maize *Adh 2* cDNA clone established that the clone hybridized with barley mRNA comparable in size to maize *Adh 2* mRNA, and that the level of this barley mRNA increased 15- to 20-fold after 1 day at 5% or 2% O_2, and about 100-fold after 1 day at 0% O_2.

We conclude that in aleurone layers, expression of the three barley *Adh* genes is maximal in the absence of O_2, that regulation of mRNA level is likely to be a major controlling factor, and that whereas the ADH system of barley has strong similarities to that of maize, it also has some distinctive features.

Aleurone layers of barley (cv Himalaya) are highly tolerant of O_2 deficit, in part because they are capable of ethanol glycolysis immediately upon transfer to anoxic conditions (11). Essential to this capability is a high constitutive ADH[3] activity (11, 13). During prolonged anoxia, a capacity for lactate glycolysis develops, but ethanol glycolysis remains important, and additional ADH activity is induced (10, 11). Recent progress in the genetics of the barley ADH system and in the molecular genetics of the related maize ADH system make possible a detailed investigation of the constitutive and anaerobically induced components of ADH in barley aleurone layers.

The ADH systems of all cereals studied (*e.g.* 1, 5, 12, 14) share many features with that of maize, which is very well characterized from the standpoints of both classical and molecular genetics (8, 9). In all cases, the active ADH isozymes are dimers, whose monomer subunits can be the products of the same or different genes. Whereas maize has two *Adh* genes which are unlinked, in barley, there are three *Adh* genes: *Adh 1*, *Adh 2*, and *Adh 3*. The *Adh 1* and *Adh 2* loci are tightly linked and sited on chromosome 4 and the *Adh 3* locus is freely recombining with *Adh 1* and *2* (2, 10, 14). The respective polypeptide products of the three barley *Adh* genes (here designated ADH 1, ADH 2, ADH 3) can combine to give three homodimers (*e.g.* ADH 1.ADH 1) and three heterodimers (*e.g.* ADH 1.ADH 2). In cv Himalaya, these six isozymes give the following pattern in native starch or polyacrylamide gels after specific staining for enzyme activity:



Scheme I

All six isozymes are titrated by sheep antiserum raised against purified maize ADH 1.ADH 1 homodimer (10).

In barley, as in maize, all the ADH isozymes can be found in anaerobically treated root tissues (14, 21, 22) and seed tissues (10). Again, as in maize, the embryo and aleurone tissues of dry seeds and aerobically germinating seeds have high constitutive levels of the ADH 1.ADH 1 homodimer (10, 12).

In the work reported here, we asked the following questions concerning O_2 deficits and the ADH system in barley aleurone layers: (a) How does the constitutive ADH 1.ADH 1 homodimer activity of the mature seed originate? (b) Does the induction of ADH isozymes by O_2 deficit reflect increases in synthesis of some or all of the three types of ADH polypeptides? (c) Does O_2 deficit increase the levels of some or all *Adh* mRNA species?

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[3] Abbreviations: ADH, alcohol dehydrogenase; LDH, lactate dehydrogenase; PDC, pyruvate decarboxylase; ANPs, anaerobic polypeptides; Urea-IEF, isoelectric focusing in the presence of 8 M urea.

MATERIALS AND METHODS

Plant Material. Aleurone layers from mature cv Himalaya barley grains were prepared and incubated in various O_2 tensions as described elsewhere (11) except that all incubations were at 22 ± 1°C on a rotary shaker (60 cycles/min). The number of layers was raised to 20 per flask when the layers were to be used as a source of ADH for column chromatography.

To obtain immature aleurone layers, plants of cv Himalaya were grown in soil either in natural daylight in the Canberra Phytotron under a 21°C day/16°C night regime, or in similar conditions in a glasshouse. Aleurone layers were prepared from developing grains by removing the embryo and distal portions of the grain and gently scraping the adhering starchy endosperm from the outer grain tissues. In grains that were rapidly accumulating ADH, microscopic examination showed that the pericarp and testa had atrophied. Dry weights of developing grains were determined after freezing overnight at −15°C and drying to constant weight at 70°C.

Extraction and Assay of ADH. ADH activity was extracted from batches of five immature aleurone layers by grinding in 2 ml of 0.15 M Tris·HCl buffer (pH 8.0) containing 10 mM DTT (11). Routine ADH assays were performed in the ethanol→acetaldehyde direction, using the assay mixture given previously (11). ADH activities are reported in IU (μmol NADH/min). In one experiment, ADH was assayed spectrophotometrically in the acetaldehyde→ethanol direction as well, in a 3-ml reaction mix containing 1 ml of 0.5 M Tris·HCl (pH 8.0), 400 μg NADH, and 30 μmol acetaldehyde.

Native Starch Gel Electrophoresis and PAGE. Horizontal starch gel electrophoresis was carried out as described by Hanson and Brown (10). Native slab PAGE was performed under the conditions described for lactate dehydrogenase (11), except that the stacking gel contained 63 mM Tris·HCl (pH 6.7). Samples for native polyacrylamide gels were ground (10 layers/ml) in 0.15 M Tris·HCl (pH 8.0) containing 10 mM DTT and clarified by centrifugation; gel tracks contained 60 μl of cleared extract plus 10 μl of 90% glycerol containing 0.1% Bromophenol-Blue. Gels were stained for ADH activity as previously described (10).

Separation of ADH Isozymes by Chromatofocusing. All operations were carried out on ice or in a cold room. ADH was extracted from batches of 250 aleurone layers by grinding with sand in a pestle and mortar in a total of 9 ml of 25 mM imidazole·HCl buffer (pH 7.4) containing 10^{-4} M $ZnSO_4$ (start buffer) plus 10 mM DTT. After centrifugation at 17,000*g* for 15 min, the supernatant was pumped (0.4 ml/min) onto a 1- × 25-cm column of PBE 94 (Pharmacia) previously equilibrated with start buffer. The column was developed at the same flow rate with 9-fold diluted Polybuffer 74 (Pharmacia) titrated to pH 4.0 with HCl and supplemented with 10^{-4} M $ZnSO_4$.

Purification of Barley ADH 1.ADH 1 Homodimer. The procedure was that developed by Pryor and Huppatz (24) for maize ADH with the following modifications. (a) All steps were carried out in a cold room. (b) Barley seed meal (400 g) was extracted twice with 0.1 M K-phosphate (pH 7.5) (1.0 L, then 0.75 L) before $(NH_4)_2SO_4$ precipitation. (c) After the chromatofocusing step on PBE 94, fractions containing ADH activity were concentrated to 6 ml using an Amicon Ultra-filtration cell with a YM-30 membrane, and then loaded onto a 2.5- × 30-cm column of Sephadex G 200 (Superfine). The column was equilibrated and eluted (0.15 ml/min) with 15 mM Na-phosphate (pH 6.5) containing 1 mM β-mercaptoethanol. The ADH activity was eluted between about 75 and 85 ml of effuent and was applied directly to the NAD^+ affinity column (Matrex Blue A, Amicon). Inclusion of this gel filtration step raised the specific activity prior to the affinity step by a factor of 4. (d) The washing buffers for the Matrex Blue A column were 15 mM Na-phosphate (pH 6.5), followed by 50 mM Na-phosphate (pH 8.0). ADH was specifically eluted by adding 4 mM NAD^+ to the second buffer, dialyzed overnight against 4 × 1 L of 50 mM Tris·HCl (pH 7.4) containing 1 mM β-mercaptoethanol, and then made 10% (v/v) with respect to glycerol. The barley ADH purified in this way was homogeneous on SDS-PAGE, and showed only ADH 1.ADH1 homodimer activity on starch gel electrophoresis. The preparation was stable for at least 10 d at 4°C in the presence of 0.03% NaN_3, and did not lose activity upon freezing at −15°C and thawing. Final recovery of enzyme (340 IU, about 2 mg protein) was comparable to that reported for maize (24).

***In Vivo* Labeling Experiments.** Sterile conditions were maintained throughout. For experiments with low specific activity [^{35}S]methionine, batches of 10 aleurone layers were first incubated in various O_2 tensions for 7 to 8 h, then rinsed thoroughly in distilled H_2O, working under an N_2 atmosphere for all treatments except air. The batches of layers then received 2 ml of fresh medium containing 20 μmol L-methionine and 0.7 or 1.0 μCi of L-[^{35}S]methionine (>800 Ci/mmol, Amersham). Layers were returned to various O_2 tensions and incubated for a further 20 h. After washing for 2 × 10 min in 20 ml of ice-cold 1 mM L-methionine to remove unabsorbed label, the layers were then ground in a total of 8 ml of Tris·HCl buffer (pH 8.0) containing 10 mM DTT. After low-speed centrifugation to pellet debris, the supernatant was centrifuged at 10°C for 2 h at 150,000*g* in a fixed angle rotor. An aliquot of the high-speed supernatant was taken for estimation of ^{35}S uptake by scintillation counting. Proteins were precipitated from the remaining supernatant by adding 4 volumes of cold acetone and holding for several hours at −15°C. Acetone precipitates were washed with 80% acetone containing 0.2 mM L-methionine until radioactivity in the washes declined to <70 cpm/ml (four washes of 5–10 ml each). The washed precipitates were then solubilized in SDS-sample buffer (28) and samples taken for scintillation counting.

Experiments with high-specific activity [^{35}S]methionine were performed as above up to the point of tissue extraction, except that batches of 10 layers received approximately 50 μCi of L-[^{35}S]methionine (>800 Ci/mmol) with no unlabeled methionine. Batches of 10 layers were extracted by grinding in 1 ml of 50 mM Tris·HCl buffer (pH 7.4) containing 150 mM NaCl, 2 mM L-methionine (NT buffer) supplemented with 0.05% v/v Nonidet P40 (NP-40) and 10 mM DTT. After low speed centrifugation to remove debris, the supernatant was centrifuged at 10°C for 1 h at 100,000*g* in a swingout rotor. An aliquot (0.1 ml) of the high-speed supernatant was used for precipitation of soluble proteins with 4 volumes of cold acetone; the pellet was washed, solubilized, and sampled for scintillation counting essentially as described above, and portions were analyzed by SDS-PAGE.

The remainder of the supernatant was taken for immunoprecipitation of ADH peptides, using antiserum raised in sheep against purified maize ADH 1.ADH 1 homodimer (24). The following criteria established that this antiserum preparation recognized all barley ADH isozymes, and hence could be used to quantitate the barley ADH peptides. (a) In immunotitration analyses of barley aleurone extracts containing all six isozymes, the ADH activity could be quantitatively removed from the supernatant by the anti-maize ADH serum (1 μl serum ≡ 0.08 IU barley ADH). (b) In native starch gels of such aleurone extracts reacted with excess antiserum, no ADH isozyme bands were detected. (c) When all three barley ADH peptides were labeled with [^{35}S]methionine, SDS-polyacrylamide gel separation of the supernatant and immunoprecipitate fractions obtained by treatment with excess antigen indicated that no labeled band corresponding to ADH peptides remained in the supernate.

Immunoreactive peptides were isolated using the *Staphylococcus aureus* protein A-antibody adsorption technique of Kessler (19). *S. aureus* cells were prepared according to Kessler (19); within 1 d of use, frozen cells were thawed, washed in NT buffer plus 0.5% NP40, then in NT buffer plus 0.05% NP40 and were

brought to a 10% suspension in the latter. Before the specific immunoprecipitation step with anti-ADH serum, a nonspecific precipitation using sheep anti-ovalbumin serum was used. Thus, to 0.5 ml of high-speed supernatant was added 3 μg of pure ovalbumin followed by 20 μl of anti-ovalbumin serum (slight antibody excess). After incubation at room temperature for 15 min, 200 μl of *S. aureus* cell suspension was added, followed by a further 15 min at room temperature. The pelleted cells (carrying nonspecifically bound polypeptides) were harvested by centrifugation at 2000*g* for 5 min and washed as described below. The supernatant from this step then received 20 μl of anti-ADH serum (about 30% antibody excess) and, after 15 min at room temperature, a further 200 μl of *S. aureus* cell suspension. After standing for 15 min at room temperature, these cells (carrying immunoprecipitated ADH peptides) were pelleted, and with the cells from the nonspecific precipitation were washed three times in 0.5 ml NT buffer plus 0.05% NP40, twice in water, and then resuspended in 0.5 ml of water. An aliquot of the suspension was taken for scintillation counting, and the remainder was analyzed by SDS-PAGE and urea-IEF as described below.

RNA Isolation and *In Vitro* Translation. Total RNA was isolated by the method of Higgins *et al.* (16), starting from batches of 40 aleurone layers incubated for 24 h in various O_2 tensions. RNA samples were translated in the wheat germ system (15) in the presence of [^{35}S]methionine. Following translation, a portion of each reaction mix was used for precipitation of total proteins by addition of 4 volumes of cold acetone. Protein was pelleted by centrifugation and dissolved in SDS-sample buffer for analysis by SDS-PAGE. The remaining reaction mix was taken for immunoprecipitation of ADH peptides, using the double antibody method. First, each mix was adjusted to 0.7 ml with 30 mM Na-phosphate buffer (pH 7.4) containing 0.45 M NaCl, 2 mM L-methionine, and 1% v/v Tween (3 × PBS-Tween), and centrifuged for 10 min at 17,000*g*. The supernatant was divided in half; one-half received 10 μl of anti-ADH serum, and the other half received 10 μl of nonimmune serum (sheep anti-ovalbumin) as a control. After 1 h at 25°C, 40 μl of rabbit antisheep IgG serum was added to both. After incubation for a further 1 h at 25°C and overnight at 4°C, the immunoprecipitates were harvested by centrifugation, washed three times in 3 × PBS-Tween, twice in water, and analyzed by urea-IEF.

SDS-PAGE and Urea-IEF. SDS-PAGE was used to separate polypeptides labeled *in vivo* and *in vitro*, and labeled bands were detected by fluorography as described elsewhere (11); when immunoprecipitated samples containing *S. aureus* cells were run, cells were first removed by centrifugation. In some gels, ^{35}S in ADH peptides was quantified by cutting out gel zones, treating with NCS Tissue Solubilizer for 4 h at 50°C, and scintillation counting.

Immunoprecipitate samples were prepared for urea-IEF as follows. Precipitates obtained by the *S. aureus* cell method or by double antibody precipitation were suspended in a known volume of water (*e.g.* 15 μl) and brought to about 8 M urea by adding 2 volumes of freshly prepared 12 M urea held at 37 to 39°C. *S. aureus* cells were eliminated by centrifugation at this stage. Samples (40 μl) of the immunoprecipitate in 8 M urea were then mixed with 5 μl of 87% glycerol and 5 μl of a stock containing 0.1 ml β-mercaptoethanol, 0.2 ml of NP-40, and 0.7 ml H_2O. After vortex mixing for 1 min, the mixture was frozen in liquid N_2 and held in this state for up to 3 h before thawing, re-mixing, and re-centrifuging briefly to defoam. The entire 50 μl sample was loaded into a urea-IEF gel track.

IEF gels (1.3 mm thickness, nominal pH range 3.5–9.5 over a 10-cm width) were prepared according to LKB Application Note 250, except that 8 M urea was included. Gels were run at 14°C using an LKB 2117 Multiphor apparatus. Gels were prefocused for 3 h (about 1.2 kv-h) after which sample wells (8 × 4 mm) were cut into the gel 2 cm from the cathode. Samples were added to the wells and the gels were then focused for 4 h (about 3.5 kv-h). Radioactivity was detected by fluorography and for *in vivo*-synthesized ADH peptides, the proportions of the individual peptides were determined by densitometry of fluorographs.

Northern Hybridization Analysis. Total RNA samples (15 μg) were electrophoresed in 2.2 M formaldehyde-1% agarose gels (modified from Ref. 20) and transferred to nitrocellulose paper according to Thomas (30). A maize *Adh 2* cDNA clone (pZml 841) was used as a hybridization probe, essentially as described by Gerlach *et al.* (9). The extent of hybridization was quantified by densitometry.

RESULTS

Developmental Origin of the Constitutive ADH Activity of Mature Aleurone Layers. The constitutive ADH of mature, dry aleurone tissue and imbibed, aerobically incubated aleurone layers is known to be almost all ADH 1.ADH 1 homodimer with a trace of ADH 1.ADH 2 heterodimer (10, 12). This endowment of ADH was accumulated during the later part of grain growth, with accumulation continuing until grains dried down (Fig. 1). Rapid ADH accumulation started at about the same time as Chl loss from the seed coat began. Because the maximum activity reached in layers before drying was close to that measured in both aleurone tissue of air-dried seeds (Fig. 1) and mature aleurone layers imbibed aerobically (11), the bulk of the ADH accumulated during grain growth must be stable during dehydration, dry-storage, and rehydration.

The isozyme composition of ADH extracted from developing aleurone layers showed two interesting features (Fig. 1, inset). First, at no stage were any isozymes containing the ADH 3 monomer detected, although isozymes containing the ADH 2 monomer were readily detected, especially early in development. This apparent difference in expression of the *Adh 2* and *Adh 3* genes has been noted in another cultivar, Proctor (14). Second, the ADH 2.ADH 2 homodimer band tended to stain more weakly as grain development progressed as did the ADH 1.ADH 2 heterodimer. Taken with the absence of an ADH 2.ADH 2 band in dry seeds and imbibed aleurone layers (10, 12), this suggests that ADH 2.ADH 2 and ADH 1.ADH 2 activities are selectively lost during grain maturation.

Induction of ADH Isozymes at Low O_2 Tensions. In mature aleurone layers, O_2 deficit increases total ADH activity by up to 60% (11), and induces five isozyme bands (Fig. 2; see also Ref. 10). These five bands contain monomers specified by all three *Adh* genes. The ADH 2.ADH 2 and ADH 3.ADH 3 homodimers showed a small but consistent difference in response to low O_2 levels: the latter stained less intensely than the former at 5% O_2, but about equally at 2% and 0% O_2 (Fig. 2).

From qualitative isozyme staining on gels, it was impossible to tell whether the ADH 1.ADH 1 homodimer increased or decreased during O_2 deficit. Thus, the ADH activity extracted from equal numbers of aerobically and anaerobically incubated layers was quantitated after fractionation on a chromatofocusing column (Fig. 3). The ADH sample from layers incubated in N_2 gave three peaks, the components of which were identified by PAGE: peak I contained ADH 1.ADH 1 homodimer only; peak II contained the heterodimers ADH 1.ADH 3 and ADH 1.ADH 2; peak III contained homo- and heterodimers of ADH 2 and ADH 3. As expected, the profile from layers incubated in air lacked peak III and had a very small peak II. Because ADH activity applied to the column was quantitatively recovered (>98%), it was possible to apportion the N_2-induced component of ADH activity among the three peaks as follows. The net increase in ADH activity following induction under N_2 was 12.9 IU; of this, 7.7 IU was contributed by peak I, 4.2 IU by peak II, and 1.0 IU by peak III. This result implies a net increase in the supply of ADH 1 monomers, as well as ADH 2 and ADH 3 monomers, during N_2 treatment.

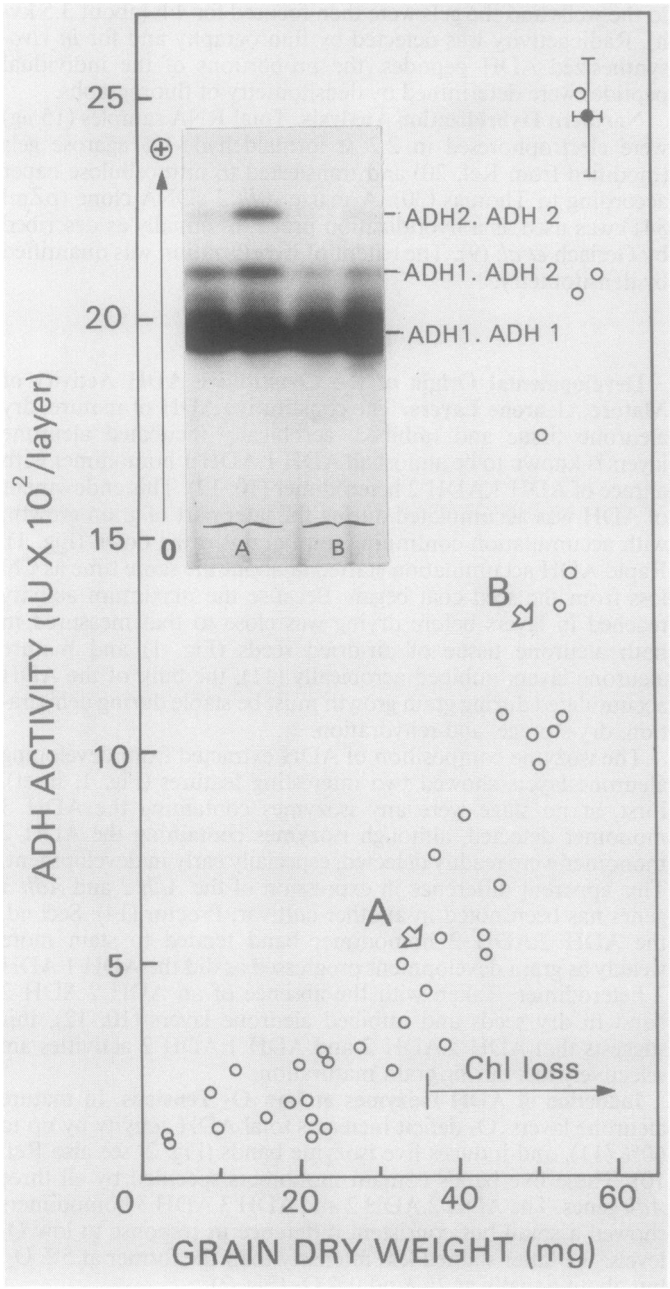


FIG. 1. Changes in ADH activity and isozyme composition during grain development of barley cv Himalaya in the Canberra Phytotron. Each open circle represents values for a batch of 10 grains from the central spikelets of a single ear; five grains were pooled for ADH assay, five for dry weight determination. Observations cover the range from about 1 to 5 weeks postanthesis. The solid point indicates the mean values (±SE) for aleurone ADH activity and dry weight for mature, air-dry seeds. The inset shows a typical starch gel zymogram for aleurone layers in the early (arrow A) and late (arrow B) phases of ADH accumulation. Each track represents a pair of aleurone layers taken from grains in the same ear. The samples were overstained to demonstrate the absence of isozymes containing ADH 3 monomers. The faint band just ahead of the broad ADH 1.ADH 1 band is a derivative (conformer) related to ADH 1.ADH 1 (10).

When the curves of Figure 3 are considered in relation to the isozyme patterns of Figure 2, it can be seen that none of the five ADH isozymes which appear on gels after O_2-deprivation can augment the total ADH activity by more than about 5 to 10%, and in the cases of the peak III isozymes, by much less than this. We eliminated the possibility that our routine ADH assay method (ethanol→acetaldhyde, the physiological reverse direction) grossly downplayed the catalytic potential of the anaerobically induced isozymes by assaying extracts from a typical time course experiment (0, 1, 2, 3 d in N_2) in both directions. During such an experiment (*e.g.* Ref. 11; Fig. 4), the contribution of the induced isozymes increases. Activity in the forward direction (y) was highly correlated with that in the reverse direction (x) (r = 0.96, significant at $P < 0.01$), and the relationship between y and x approximated 4:1.

***In Vivo* Protein Synthesis.** The types and amounts of ADH polypeptides being synthesized *de novo* in various O_2 tensions were derived by combining the results of two types of labeling experiments. Experiments with substrate levels of low-specific activity [^{35}S]methionine gave estimates of the amount of total soluble polypeptides synthesized. Experiments with tracer levels of high-specific activity methionine in which the ADH polypeptides were immunoprecipitated and analyzed by SDS-PAGE and urea-IEF gave estimates of each ADH polypeptide as a percentage of total soluble [^{35}S]polypeptide synthesis.

Low-Specific Activity Experiments. Figure 4 shows that the amount of [^{35}S]methionine taken up by the aleurone tissues (≥200 nmol/layer) was large compared to the size of the endogenous pool of free methionine (<20 nmol/layer), and in relation to methionine consumption in protein synthesis during the experiment. Under such conditions, data on ^{35}S incorporation into polypeptides can furnish quantitative estimates of protein synthesis. Thus, Figure 4 indicates that the total soluble ^{35}S-polypeptide synthesis declined progressively as O_2 deficit increased, so that in 0% O_2 it was only about 11% of that in air.

High-Specific Activity Experiments. As O_2 deficit was increased, the profile of labeled polypeptides separated by SDS-PAGE altered, some bands decreasing in intensity while others increased. The percentage of the profile accounted for by immunoprecipitated ADH increased markedly (Fig. 5A). Note that all the barley ADH peptides have similar molecular weights (M_r ~42,000) and that they run together on SDS-PAGE as a diffuse double band. For the experiment of Figure 5A, the percentages of ADH peptides in the total profiles were as follows: 21% O_2, 0.61%; 5% O_2, 1.3%; 2% O_2, 3.5%; 0% O_2, 13.8%. For immature aleurone layers from developing grains that were incubated with [^{35}S]methionine in air (not shown in Fig. 5A), the incorporation into ADH was 0.75% of the total.

Urea-IEF was used to separate the individual immunoprecipitated ADH peptides (Fig. 5B). The positions occupied by the ADH 1, ADH 2, and ADH 3 monomers on urea-IEF gels have been previously established by analyzing the ADH peptides of electrophoretic variants at the three *Adh* loci (10). Additional evidence confirming the identity of the labeled ADH 1 band was obtained in the present study from the co-migration of the labeled band with unlabeled polypeptide from highly purified barley ADH 1.ADH 1 homodimer (Fig. 5B, arrow). The minor band designated as ADH 1′ in Figure 5B is considered from genetic evidence to be a posttranslational modification of the *Adh 1* gene product (10). Two other minor bands, labeled CRM+ in Figure 5B could not be identified with any of the three known *Adh* genes (10), but were shown to cross-react with ADH 1.ADH 1 homodimer as follows. An ^{35}S-labeled enzyme preparation containing all six ADH isozymes was supplemented with 2-, 10-, and 20-fold excesses of unlabeled purified ADH 1.ADH 1, and was then mixed with sufficient anti-ADH serum to titrate only the labeled isozymes. The antigen-antibody complexes were precipitated with *S. aureus* cells and analyzed by urea-IEF. At increasing antigen excess, the label was competed out of the two unidentified minor bands just as it was from the ADH 1, ADH 1′, ADH 2, and ADH 3 bands (results not shown).

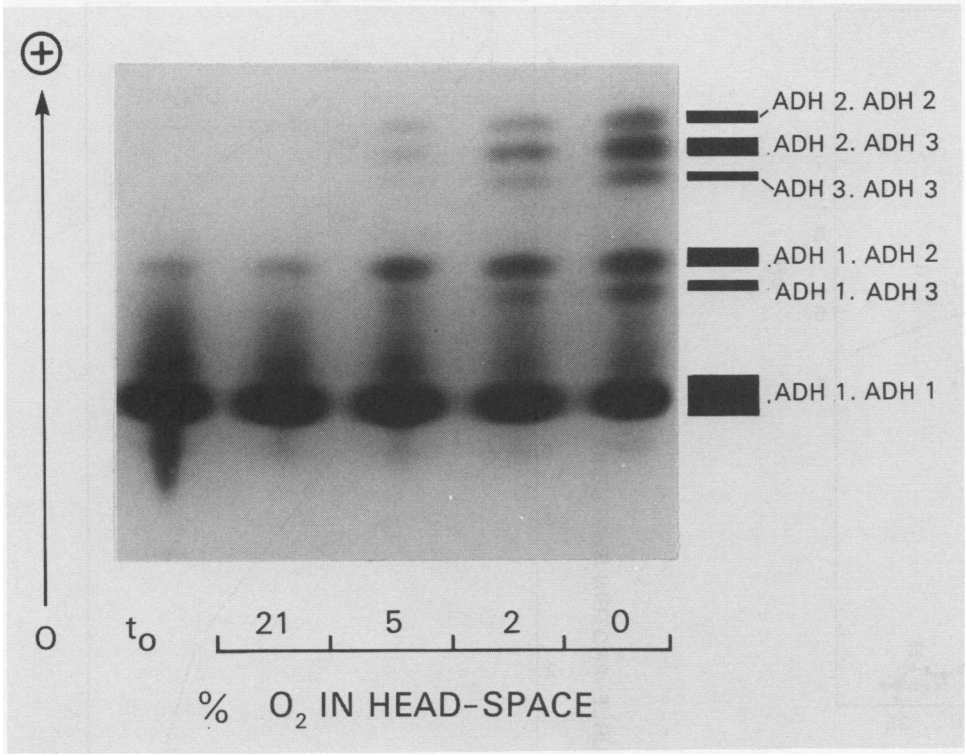


FIG. 2. The effect of incubation for 1 d in various O_2 tensions on the ADH isozymes of barley aleurone layers. Isozymes were separated by native PAGE. The first track (t_0) shows the pattern for freshly prepared layers before incubation. The subunit composition of the ADH dimers according to Harberd and Edwards (14) is shown on the right.

Figure 5B shows that in air ADH 1 and its presumed derivative ADH 1′ accounted for almost all the radioactivity, although there was also a faint band in the ADH 2 position. The pattern in 5% O_2 differed from that in air only by a small increase in the proportion of ADH 2, but at 2% O_2 there were much larger differences from air: a prominent ADH 3 band appeared, and the ADH 2 band strengthened markedly. At 0% O_2, the ADH 3 and ADH 2 peptides became still more prominent relative to ADH 1.

Figure 5B also shows the ADH peptides produced by immature aleurone layers stripped from developing grains and incubated with [^{35}S]methionine in air. The pattern is very similar to that of mature layers in air in the adjacent gel track.

Isolation and *In Vitro* Translation of Total RNA. The yield of RNA from batches of 40 layers was approximately 150 μg. Although yields varied by a factor of two, there was no trend towards lower RNA recovery at low O_2 tension.

On SDS-PAGE, the *in vitro* translation products of RNA isolated from layers incubated at all O_2 tensions had many common features, but several bands appeared or strengthened as the O_2 concentration decreased (Fig. 6A). Among the bands which strengthened, that in the zone characteristic of ADH peptides was very prominent. There is a contrast between the *in vitro* polypeptide synthesis pattern of Figure 6A, in which many bands are added or strengthened at lower O_2 tensions, and the *in vivo* pattern of Figure 5A, in which many bands become weaker while others strengthen at lower O_2 tensions.

Labeled polypeptides immunoprecipitated from total translation products, and then separated by urea-IEF are shown in Figure 6B, for the 0% O_2 treatment only. (The other three treatments gave immunoprecipitates containing insufficient label for analysis.) In the 0% O_2 treatment, sharp bands identical in position to *in vivo* synthesized ADH 1, ADH 2, and ADH 3 peptides were present, together with a novel, sharp band ahead of ADH 1; there was no band in the usual ADH 1′ position. There was also a diffuse band not seen in *in vivo* labeled samples, cathodal to the ADH 1 position. Since no bands were seen in control samples prepared with nonimmune serum (not shown), the novel bands of Figure 6B may be incomplete ADH peptides which share some antigenic determinants with *in vivo* synthesized ADH monomers.

Northern Hybridization Analysis. In all samples, the maize *Adh 2* genomic clone used as a probe recognized an RNA species of a size comparable to maize *Adh 2* messenger (Fig. 7). For equivalent amounts of total RNA, the extent of hybridization in 5% O_2 and 2% O_2 samples was between 15- and 20-fold greater than in the air sample and some 100-fold greater in the N_2 sample. The hybridization observed cannot be ascribed to any particular barley *Adh* mRNA because the extent of homology of the various barley *Adh* mRNA species with the maize *Adh 2* clone is unknown.

DISCUSSION

The ADH system of barley clearly resembles that of maize with respect to polypeptide composition, mRNA structure, and regulation of gene expression during anaerobiosis. In maize, the tissue chosen for studying the anaerobic induction of ADH has been the primary root, which can survive up to 3 d of anoxia (27). By comparison, primary roots of barley are less tolerant of prolonged anoxia than maize roots (10, 21). On the other hand, barley aleurone layers not only survive well during complete O_2 deprivation (11), but actually require this condition for maximum expression of the *Adh* genes. Thus, aleurone layers of barley are likely to prove useful in further studies of ADH in particular, and ANPs in general.

Since the overall similarity to the maize ADH system is so great, the following discussion emphasizes the differences between the barley and maize ADH systems.

Three-Gene ADH Isozyme Pattern and *In Vivo* ADH Peptide Synthesis. The ADH isozyme profile in immature barley aleurone layers—mainly ADH 1.ADH 1 homodimer activity with much lower activities of ADH 1.ADH 2 and ADH 2.ADH 2—correlates with the *in vivo* ADH peptide synthesis pattern, which is dominated by ADH 1 and contains a trace of ADH 2. The situation in immature maize seed tissues is comparable (7, 23), with maize ADH 1.ADH 1 homodimer predominant, and traces

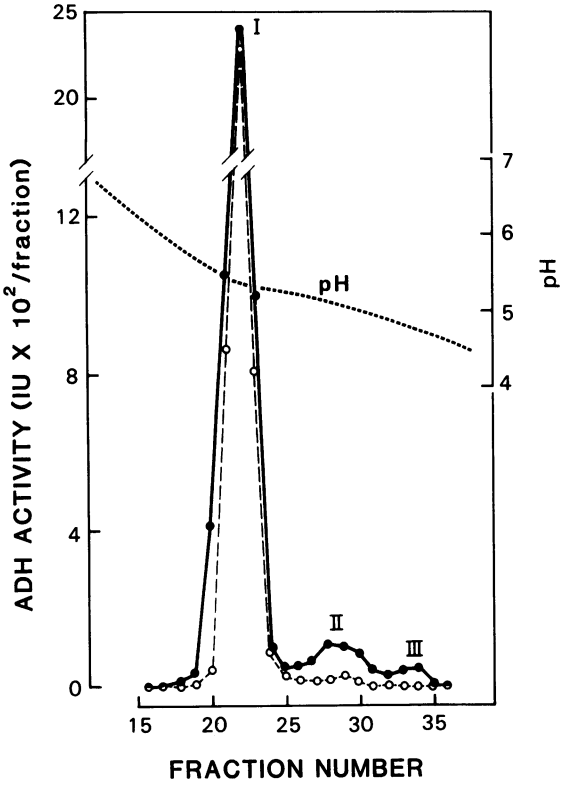


FIG. 3. Fractionation on a chromatofocusing column of total ADH activity extracted from 250 aleurone layers incubated for 2 d in air (O- - -O) or N_2 (●——●). Fraction volume was 5 ml. PAGE showed that peak I (apparent pI = 5.3) contained only ADH 1.ADH 1 homodimer, peak II (apparent pI = 5.1) mainly ADH 1-containing heterodimers, and peak III (apparent pI = 4.8) mainly homo- and hetero- dimers of ADH 2 and ADH 3; peaks II and III both contained small amounts of ADH 1.ADH 1 homodimer. Total ADH activity applied to column: air, 44.2 IU; N_2, 57.1 IU. Recovery of ADH activity from the columns was >98%.

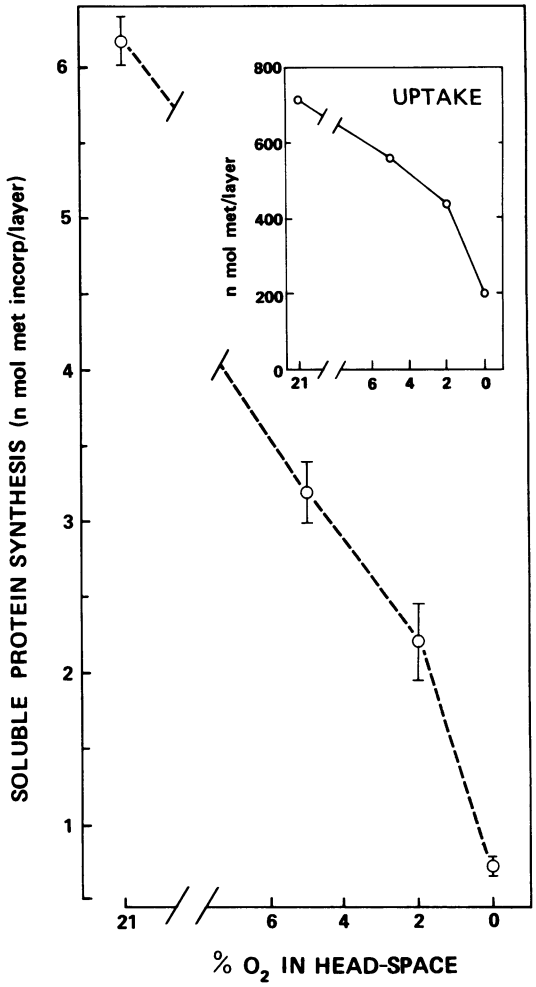


FIG. 4. The effect of O_2 tension on the uptake and incorporation into soluble protein of a large dose of low specific activity [^{35}S]methionine (0.7 or 1 μCi/μmol; 20 μmol/10 layers). Aleurone layers were incubated at the various O_2 tensions for 7 to 8 h before addition of label; incubation was then continued for 20 h. The total free α-amino nitrogen pool determined by Rosen's method (25) in layers incubated in air or N_2 was about 200 nmol/layer. TLC showed that methionine was only a minor (<10%) component of the total free amino acid pool. Data points are means (±SE) for two experiments, each with duplicate samples at each O_2 tension.

of ADH 1.ADH 2 heterodimer. However, unlike maize, barley has a third *Adh* gene (*Adh* 3) and this remains silent during seed development, at least under aerobic conditions.

In mature barley aleurone layers, the changes in ADH isozymes provoked by O_2 deficits also correlate with the patterns of *in vivo* ADH peptide synthesis, as summarized in Figure 8. This figure is a synthesis of data from low- and high-specific activity labeling experiments, giving the amounts of the three ADH peptides produced at each O_2 level and depicting in the inset the isozyme profiles which could be generated by random dimerization of the ADH monomers produced at each O_2 level. Note that the inset data refer only to peptides synthesized during a 24-h period and so do not show the large amount of ADH 1.ADH 1 homodimer carried over from seed development. When allowance for this initial endowment of ADH 1.ADH 1 homodimer is made, the predictions of Figure 8 are in broad agreement with the experimental data of Figure 2. Such a result suports the notion that the change in the isozyme pattern results from random association between newly synthesized polypeptides and does not involve recycling of ADH 1 monomer from previously formed dimers.

A direct correspondence between ADH peptide synthesis and anaerobic ADH induction is also well established for maize roots (6, 26). In maize roots, neither ADH 1 nor ADH 2 are significantly labeled in air, and the ratio of ADH 1:ADH 2 labeling (with [^{3}H]leucine) is about 2:1 under argon (27). As shown in Figure 8 (and by the primary data of Fig. 5), the situation in barley aleurone layers differs in several respects from that in maize roots. First, there is substantial ADH 1 synthesis in aerobic conditions in barley, and a little ADH 2 synthesis. Since there is no net increase in ADH activity in air (11) this implies that the ADH 1.ADH 1 homodimer is turning over. This is considered not to occur in maize seed tissues (17). Calculations in which the methionine content of barley ADH 1 is taken to be the same as that of maize ADH 1, (12 mol methionine/mol ADH) and a specific activity of ~0.2 IU/μg is used, indicate the extent of this turnover in air to be about 10%/d. A second distinction from maize is the evidence for some processing of the primary ADH 1 polypeptide to the species we designate ADH 1′. A labeled band in the ADH 1′ position was noted in urea-IEF analyses of most barley and wild barley lines tested, and in a slow electrophoretic variant of *Adh 1* the ADH 1′ band shifted in concert with the ADH 1 band (10). Further indication of a relationship of ADH 1′ to ADH 1 comes from Figure 5B, where ADH 1′ can be seen clearly in immature and mature, aerobic samples that contain little or no ADH 2 or ADH 3. A posttranslational origin for ADH 1′ is suggested by the lack of a band in the usual

ADH 1′ position among the *in vitro* synthesized ADH peptides (Fig. 6B). A third distinctive feature of the barley system is a small differential induction of the ADH 2 and ADH 3 peptides

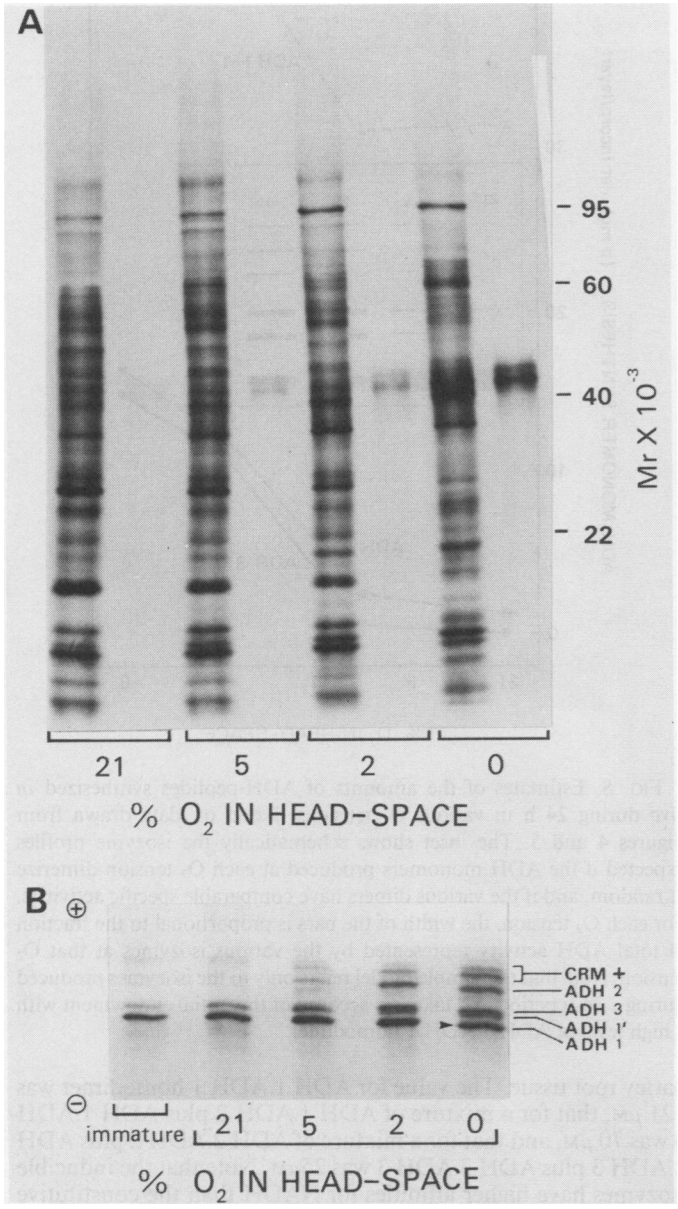


FIG. 5. Fluorographs of gel separations of labeled polypeptides synthesized *in vivo* using high specific activity [^{35}S]methionine (50 μCi/10 layers). Other conditions for labeling as in Figure 4. A, SDS-PAGE separations of total soluble polypeptides (left-hand track of each pair) and the polypeptides precipitated by anti-ADH antiserum followed by *S. aureus* cells (right-hand track of each pair). Controls challenged with nonimmune serum gave no labeled precipitate. The total polypeptide tracks contained approximately equal amounts of acetone-precipitable radioactivity. B, Urea-IEF separations of the immunoprecipitated polypeptides shown in A, and also of the labeled polypeptides precipitated by anti-ADH antiserum from extracts of immature aleurone layers labeled in air with [^{35}S]methionine as above. The developing seeds from which these layers were taken were at about stage B in Figure 1, and came from glasshouse-grown plants. Arrow marks the position of the Coomassie-blue stained band found when approximately 3 μg of purified ADH 1.ADH 1 homodimer was mixed with the 0% O_2 sample. All the ADH peptides are identified on the right. Tracks contained 2.9 to 4.4 μCi of [^{35}S]polypeptides.

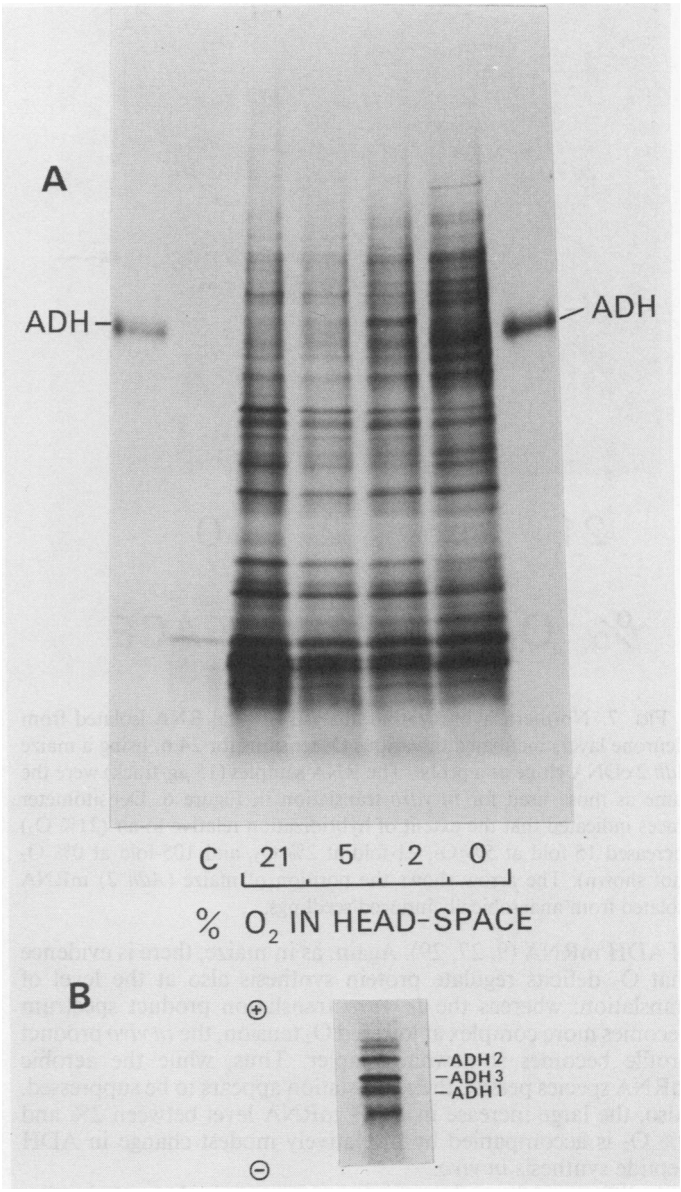


FIG. 6. Fluorographs of gel separations of ^{35}S-labeled polypeptides synthesized *in vitro* using total RNA isolated from aleurone layers incubated for 24 h in various O_2 tensions. A, SDS-PAGE separations of total translation products. Track C is a control with no added RNA. All translation product tracks except C contained approximately equal amounts of TCA-precipitable radioactivity. The outermost tracks are standards of *in vivo* synthesized ADH polypeptides; *in vitro* synthesized ADH peptides ran in the same position (not shown). B, Urea-IEF separation of the labeled polypeptides precipitated from the *in vitro* translation products of the 0% O_2 sample, using sheep anti-ADH antiserum followed by rabbit antisheep IgG. Positions occupied by *in vivo* synthesized markers run in an adjacent track are shown on the right.

and their respective isozymes in moderate O_2 deficits. Thus, at 5% O_2, ADH 2 synthesis is several-fold greater than ADH 3 synthesis, but this difference disappears at 2% and 0% O_2.

***In Vitro* Protein Synthesis and ADH mRNA Levels.** The results of *in vitro* protein synthesis show that O_2 deficit elicits an increase in the level of translatable mRNA for all ADH peptides, and the hybridization analysis demonstrates that this is accompanied by an increase in the abundance of RNA sequences recognized by a relevant DNA probe. Thus, as in maize roots, levels of ADH peptide synthesis are determined in part by levels

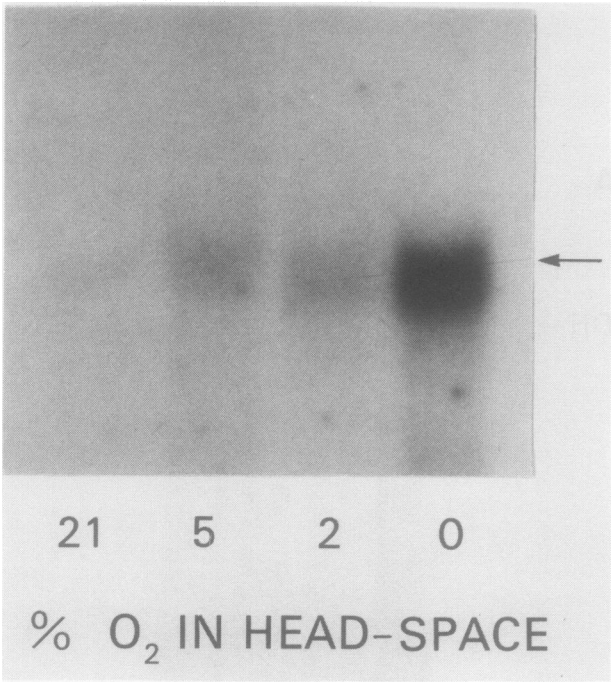


FIG. 7. Northern hybridization analysis of total RNA isolated from aleurone layers incubated in various O_2 tensions for 24 h, using a maize *Adh 2* cDNA clone as a probe. The RNA samples (15 μg/track) were the same as those used for *in vitro* translation in Figure 6. Densitometer traces indicated that the extent of hybridization relative to air (21% O_2) increased 15-fold at 5% O_2, 21-fold at 2% O_2, and 105-fold at 0% O_2 (not shown). The arrow shows the position of maize (*Adh 2*) mRNA isolated from anaerobically induced seedlings.

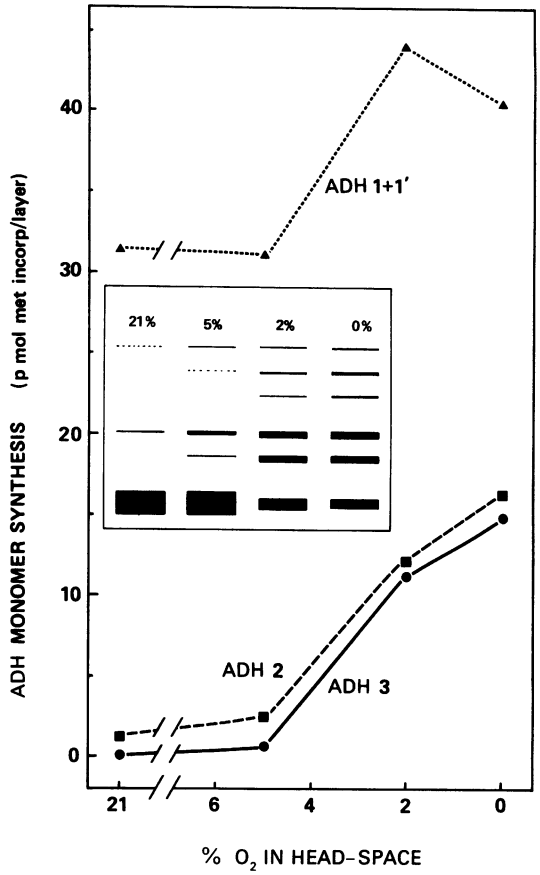


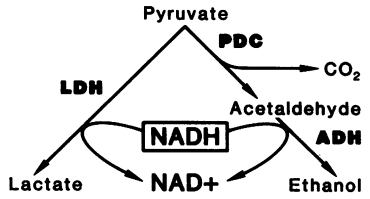
FIG. 8. Estimates of the amounts of ADH-peptides synthesized *in vivo* during 24 h in various O_2 tensions. Based on data drawn from Figures 4 and 5. The inset shows schematically the isozyme profiles expected if the ADH monomers produced at each O_2 tension dimerize at random, and if the various dimers have comparable specific activities. For each O_2 tension, the width of the bars is proportional to the fraction of total ADH activity represented by the various isozymes at that O_2 tension. Note that this simple model refers only to the isozymes produced during a 24-h period and takes no account of the initial endowment with a high level of ADH 1.ADH 1 homodimer.

of ADH mRNA (9, 27, 29). Again, as in maize, there is evidence that O_2 deficits regulate protein synthesis also at the level of translation: whereas the *in vitro* translation product spectrum becomes more complex at lowered O_2 tension, the *in vivo* product profile becomes somewhat simpler. Thus, while the aerobic mRNA species persist, their translation appears to be suppressed. Also, the large increase in ADH mRNA level between 2% and 0% O_2 is accompanied by a relatively modest change in ADH peptide synthesis *in vivo*.

A difference from the maize root system is that our *in vitro* translation products were directed by RNA isolated from tissues incubated for 24 h in the various O_2 tensions. Clearly, many of the original messages were still present at this time. In maize, the original translatable message population has decayed substantially by 24 h of O_2 deprivation (27). Barley aleurone tissue can function almost normally as soon as it is returned to air following 4 d under N_2 (11) but maize roots die after 3 d of anoxia (27). We speculate that the prolonged retention of a normal population of aerobic messages in barley aleurone contributes to the capacity to recover rapidly from prolonged anoxia.

Is ADH Induction during O_2 Deficit Metabolically Significant? Mature barley aleurone tissues can be considered as pre-adapted to O_2 deficit as a result of developmentally regulated expression of the *Adh 1* gene during grain growth. The mature grain is endowed with a capacity for ethanol glycolysis, which is activated at once if anoxic conditions occur after imbibition. Since the additional ADH isozymes that appear during O_2 deprivation increase the total catalytic potential of ADH by no more than 60%, it might be argued that the specific, complex response of ADH induction has little impact on ethanol glycolysis.

Two recent findings suggest an alternative interpretation. First, Mayne and Lea (22) have reported large differences in the K_m for NADH among the ADH isozymes isolated from anaerobic barley root tissue. The value for ADH 1.ADH 1 homodimer was 121 μM, that for a mixture of ADH 1.ADH 2 plus ADH 1.ADH 3 was 70 μM, and that for a mixture of ADH 2.ADH 2 plus ADH 2.ADH 3 plus ADH 3.ADH 3 was 25μM. Note that the inducible isozymes have higher affinities for NADH than the constitutive isozyme. Second, aleurone tissues show anaerobic induction of lactate glycolysis and of LDH activity; the induced LDH has a K_m for NADH of 20 μM (11), so that its affinity for cofactor is about 6-fold higher than that of the constitutive ADH isozyme, but similar to that of some of the induced isozymes. From Scheme II below, it can be seen that as LDH activity rises during O_2 deficit, lactate glycolysis begins to compete with ethanol glycolysis, both for pyruvate (4) and for NADH.



Scheme II

Thus, the higher affinities for NADH among the induced ADH

isozymes could increase the ability of ethanol glycolysis to compete with lactate glycolysis. Preserving a balance between those two pathways may be important for survival of O_2 deficit, in terms of pH regulation (4, 18) and loss of carbon to the environment (3). We speculate that the complex 3-gene ADH system of barley evolved in response to the development of an inducible LDH system, with selection pressure for balanced lactate and ethanol glycolysis favoring diversity of cofactor affinity among the ADH gene products. In this connection, it is intriguing that no inducible LDH system has so far been demonstrated in maize, which has a simpler ADH system than barley.

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