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Further studies on the alcohol dehydrogenases in barley: evidence for a third alcohol dehydrogenase locus and data on the effect of an *alcohol dehydrogenase – 1 null* mutation in homozygous and in heterozygous condition

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SUMMARY

This paper presents evidence that the alcohol dehydrogenases (ADHs) in barley are specified by three loci. Six distinct ADH isozymes are observed following native slab polyacrylamide gel electrophoresis of crude extracts from flooded wild-type roots. Three of these isozymes are missing in flooded roots of plants homozygous for the *Adhl-M9* mutation. The results also indicate that a simple binomial model (incorporating random dimerization and no inhibitive interaction of the two subunit species within heterodimers) is unable to account for the distribution of the total ADH activity between the ADH isozymes observed. Finally, the level and distribution of ADH activity in heterozygous (*Adhl+/Adhl-M9*) flooded roots is not what would be expected if these contain only one-half of the available ADH1 protomers and the same frequency of available ADH2 and ADH3 protomers as is contained in the flooded roots of wild-type homozygotes (*Adhl+/Adhl+*).

1. INTRODUCTION

Alcohol dehydrogenase (ADH, E.C.1.1.1.1.) activity is induced in flooded roots of maize and barley (Freeling, 1973; Harberd & Edwards, 1982). Previous investigations with barley indicated that, as in maize, the ADHs in immersed roots are specified by two loci, *Adh1* and *Adh2*, and that the polypeptide products of these loci (the ADH protomers) homo- and heterodimerize to form the three isozyme sets then observed (Harberd & Edwards, 1982). In this paper evidence suggesting that there are actually three loci specifying the ADHs in barley is presented.

Dimerization of ADH protomers in immersed maize roots is thought to occur at random. The Set I (ADH1.ADH1 homodimer):Set II (ADH1.ADH2 heterodimer):Set III (ADH2.ADH2 homodimer) activity ratio of 1:1·0–1·2:0·1–0·2,

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observed in one inbred line, is compatible with random dimerization provided that the ADH1 and ADH2 protomers are present in equal frequency and that the ADH2 subunits have no more than 20% of the specific activity of ADH1 subunits (Freeling, 1973). In barley the difference in specific activity between the ADH1 and ADH2 polypeptides appears to be less (Harberd & Edwards, 1982). Furthermore, the data presented below indicate that a simple binomial model of the kind apparently applicable to the maize ADHs is inadequate as a description of the mechanism by which the total ADH activity in flooded barley roots is distributed between the ADH isozyme bands observed.

Experiments investigating the effects of heterozygosity for the *Adh1 null* mutation *Adh1-M9* on the alcohol dehydrogenases in immersed barley roots are described in this paper. The purpose of these experiments was to observe the effect of such heterozygosity on the total ADH activity level within immersed roots and also on the distribution of activity between the different isozyme sets. These investigations represent an initial step towards defining the enzyme level properties of the barley ADH gene expression regulation mechanism.

2. MATERIALS AND METHODS

(i) *Plant material*

The barley (*Hordeum vulgare* cv. Proctor) used in this study was supplied by the National Seed Development Organization. The *Adh1-M9* mutation was derived from Proctor by sodium azide mutagenesis (Harberd & Edwards, 1982). Crossing was performed essentially as described by Briggs (1978). The F1 *Adh1+/Adh1-M9* heterozygous plants used in this study were all obtained through crosses of *Adh1+/Adh1+* as male pollen parent to *Adh1-M9/Adh1-M9* as female parent. These crosses were performed exclusively in this direction in order to ensure that the resulting progeny did not arise through accidental self pollination of the female parent. Any F1 individual capable of specifying the ADH1 polypeptide must be the progeny of the cross and could not have resulted from self pollination. Many of the heterozygotes used in this study were grown to seed and were observed to segregate in the F2 for phenotypically wild-type and phenotypically mutant individuals.

(ii) *Gel electrophoresis and scanning*

Native slab polyacrylamide gel electrophoresis was performed as follows. Entire flooded root systems were homogenized in 300 μl pre-chilled 50 mM-tris-HCl pH 6·8 with 5% (v/v) 2-mercaptoethanol and 12% (v/v) glycerol. The slurry was centrifuged at 10000 ***g*** for 15 min. Typically, 15 μl of sample supernatant was loaded onto a 1·5 mm thick slab gel containing 12% (w/v) acrylamide, 0·4% (w/v) *N*,*N*′-methylene-bisacrylamide (Bis), 0·44 M-tris-HCl pH 8·8 and 12% (v/v) glycerol, with a stacking gel containing 4% (w/v) acrylamide, 0·13% (w/v) Bis, 0·06 M-tris-HCl pH 6·8 and 12% (v/v) glycerol. The gels were run at 4 °C with 10 mM-tris, 77 mM-glycine electrode buffer at 8 mA overnight, stained with an

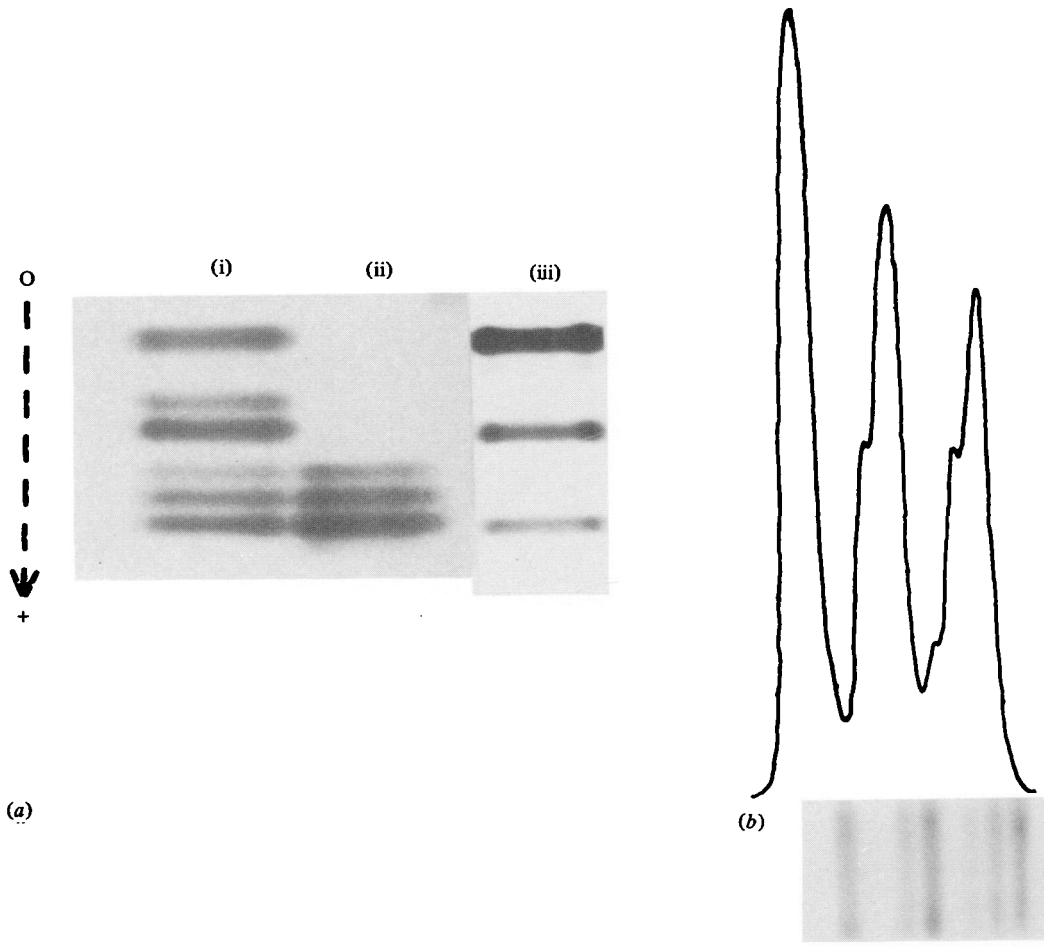

PLATE 1

(*a*) Photograph of ADH isozymes in (i) Flooded *Adh1+/Adh1+* root, (ii) Flooded *Adh1-M9/Adh1-M9* root, and (iii) developing *Adh1+/Adh1+* endosperm following separation by native slab polyacrylamide electrophoresis.
(b) Photograph and gel scanner tracing of the ADHs in flooded *Adh1+/Adh1+* roots.

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ADH stain (Harberd & Edwards, 1982) and if required scanned at 540 nm using a recording spectrophotometer with gel scanning attachment. The relative activity of each isozyme group was determined by cutting out the relevant section of the trace and estimating its area by weighing. Control experiments (data not shown) indicated that stain intensity is proportional to activity loaded over the range of activities described in this paper, and that the electrophoretic conditions employed do not result in preferential degradation of any of the isozyme groups.

(iii) *Spectrophotometric determination of ADH activity*

Spectrophotometric determinations of total ADH activity were performed at pH 10 essentially as described previously (Harberd & Edwards, 1982), except that the extraction buffer was 0·05 M-sodium phosphate pH 7·4, 10 mM-dithiothreitol and the reaction was assayed at 20 °C. Experimental pH range determinations indicated that both the ADH1 and ADH2 polypeptides are maximally active in the ethanol to acetaldehyde direction at pH 10 (data not shown). Protein concentration was determined as described by Sedmak & Grossberg (1977).

(iv) *Root flooding*

Barley roots were flooded as described previously (Harberd & Edwards, 1982). In this paper the term flooded roots refers specifically to the roots of 8-day-old plants which have been flooded for a further 4 days.

(v) *Dissociation–reasssociation conditions*

Flooded *Adh1*+/*Adh1*+ roots were homogenized in pre-chilled dissociation buffer containing 1 M-NaCl, 0·1 M-2-mercaptoethanol, 0·4 M-sucrose, 0·1 M-phosphate pH 7·0. The extract was centrifuged at 10000 ***g*** for 15 min at 4 °C, and the supernatant frozen at −20 °C for 12–15 h. The activity was zero immediately on thawing. Samples were then dialysed against a buffer containing 0·1 M-2-mercaptoethanol, 0·4 M-sucrose, 0·1 N-tris-HCl pH 7·5, 0·5 mM-$ZnCl_2$ for 9 h at 4 °C. The activity recovered following dialysis was 60–70 % of that in the original extract.

3. RESULTS

(i) Electrophoretic separation of ADH isozymes in flooded roots and developing endosperm

The ADH activity in *Adh1*+/*Adh1*+ flooded roots can be resolved as six anodally migrating bands in native polyacrylamide gel electrophoresis (Plate 1*a*). In flooded roots of plants homozygous for the *Adh1 null* mutation *Adh1-M9* (see Harberd & Edwards, 1982) only the three fastest migrating bands are observed (Plate 1*a*). In developing endosperm of *Adh1*+/*Adh1*+ seeds the first, third and sixth bands (in order from the origin) are observed but the second, fourth and fifth are not (Plate 1*a*).

The observation of six ADH bands in *Adh1*+/*Adh1*+ flooded roots rather than

the three previously recognized (Harberd & Edwards, 1982) results from the greater resolving power of the polyacrylamide gel system over the starch system. These six isozyme bands can be divided into three groups, Group A, Group B and Group C, where Group A corresponds to the Set I, Group B to the Set II, and Group C to the Set III previously observed with starch (fig. 1., Harberd & Edwards, 1982).

Fig. 1. Diagram of a three locus model accounting for the ADH isozymes in barley.

Table 1. *Mean Group A : B : C activity ratio* (±S.D., *Steel & Torrie, 1960*) *in roots of* Adh1+/Adh1+ *and* Adh1-M9/Adh1-M9 *plants, and following* in vitro *dissociation-reassociation of ADHs in* Adh1+/Adh1+ *flooded roots* (*DR* Adh1+/Adh1+)

	Group		
	A	B	C
Adh1+/Adh1+	1 :	1·133±0·059 :	1·183±0·209
Adh1+/Adh1-M9	1 :	1·137±0·147 :	1·236±0·244
DR *Adh1+/Adh1+*	1 :	1·06±0·09 :	1·20±0·24

The mean Group A : B : C activity ratio obtained by gel scanning following native polyacrylamide gel separation of extracts from flooded roots of five *Adh1+/Adh1+* homozygous plants was found to be 1 : 1·133 : 1·183, and not significantly different from 1 : 1 : 1 (Table 1). A specimen scan trace is shown in Plate 1(*b*).

(ii) *Computer predictions of the Group A : B : C ratio*

The ADH isozymes in flooded barley roots can be divided into three groups as described above (Fig. 1). Group A consists of Set I (ADH1 homodimers), Group B of Sets II and III (ADH1 containing heterodimers) and Group C of Sets IV, V and VI (non-ADH1 containing homodimers and heterodimers).

Now let p = frequency of ADH1 protomers, q = frequency of ADH2 protomers, r = frequency of ADH3 protomers and $p+q+r = 1$.

On the basis of the three locus model presented above, and on the assumption

that dimerization occurs at random, it follows that the protein level ratio for each of the isozyme Sets relative to Set I will be given by

$$\begin{matrix} \text{I} & \text{II} & \text{III} & \text{IV} & \text{V} & \text{VI} \\ p^2 : & 2pr : & 2pq : & r^2 : & 2qr : & q^2. \end{matrix}$$

Now let the specific activity of the ADH1 monomer $= a_1$, of the ADH2 monomer $= a_2$, and of the ADH3 monomer $= a_3$. Provided that the specific activity of any ADH monomer is the same in homodimers and in heterodimers the Sets I–VI activity ratios will be given by

$$\begin{matrix} \text{I} & \text{II} & \text{III} & \text{IV} & \text{V} & \text{VI} \\ a_1p^2 : & (a_1+a_3)pr : & (a_1+a_2)pq : & a_3r^2 : & (a_2+a_3)qr : & a_2q^2. \end{matrix}$$

Since Group A consists of Set I, Group B of Sets II and III and Group C of Sets IV, V and VI it follows that the Group A:B:C activity ratio will be given by

$$\begin{matrix} \text{A} & & \text{B} & & \text{C} \\ a_1p^2 : & p[(a_1+a_2)q+(a_1+a_3)r] : & a_2q^2+(a_2+a_3)qr+a_3r^2. \end{matrix} \quad (1)$$

Calculations of the expected Group A:B:C ratio from equation (1) were performed by computer for all possible combinations of p, q, r, a_1, a_2 and a_3 when $p = 0{\cdot}1,\ 0{\cdot}2 \ldots 0{\cdot}9$, $q = 0{\cdot}1,\ 0{\cdot}2 \ldots 0{\cdot}9$, $r = 0{\cdot}1,\ 0{\cdot}2 \ldots 0{\cdot}9$ such that $p+q+r = 1$, $a_1 = 1$, $a_2 = 0{\cdot}2,\ 0{\cdot}4 \ldots 2{\cdot}0$, $a_3 = 0{\cdot}2,\ 0{\cdot}4 \ldots 2{\cdot}0$ (a_2 and a_3 are relative activities of the ADH2 and ADH3 monomers with respect to a_1, the activity of the ADH1 monomer). The computer was instructed to print only those value combinations for which the predicted A:B:C fell within the limits 1 :0·5–1·5 :0·5–1·5. Twenty-nine such combinations were obtained and the mean Group A:B:C activity ratio ($\pm$standard deviation) was $1 : 1{\cdot}465 \pm 0{\cdot}025 : 0{\cdot}531 \pm 0{\cdot}014$. In all cases the generated ratios were significantly different from the ratio observed (A:B:C, $1 : 1{\cdot}133 \pm 0{\cdot}059 : 1{\cdot}183 \pm 0{\cdot}209$) indicating that equation (1), given the parameter values above is consistently inadequate as an explanation of the Group A:B:C activity ratio observed. It can also be shown, by deriving a quadratic equation for the solution of p from equation (1), that values of B and C close to unity do not fit the hypothesis since they give complex roots for p (Reeve, pers. comm.).

(iii) In vitro *dissociation and reassociation of the ADHs from flooded* Adh1 + /Adh1 + *roots*

In vitro dissociation and reassociation of the ADHs from three flooded *Adh1 + /Adh1 +* roots was performed as described above. Following dialysis they were subjected to native polyacrylamide gel electrophoresis and the Group A:B:C ratio was found to be 1 :1·06 :1·20, not significantly different from the native ratio in *Adh1 + /Adh1 +* flooded roots (Table 1).

(iv) *Comparative investigations of the ADHs in flooded wild-type homozygote* (Adh1 + /Adh1 +) *and heterozygote* (Adh1 + /Adh1-M9) *roots*

The mean Group A : B : C activity ratio following native polyacrylamide gel separation of extracts from flooded roots of five *Adh1* + /*Adh1-M9* heterozygous plants was determined and compared with those of five *Adh1* + /*Adh1* + homozygous plants (Table 1). The two ratios are not significantly different, and the relative activity of each isozyme group is constant irrespective of the genotype of the individual concerned.

Table 2. *ADH activity in flooded roots of* Adh1 + /Adh1 + , Adh1 + /Adh1-M9, *and* Adh1-M9/Adh1-M9 *plants**

Genotype	Activity/mg protein
Adh + /*Adh1* +	1·742 ± 0·245
Adh1 + /*Adh1-M9*	1·152 ± 0·062
Adh1-M9/*Adh1-M9*	0·955 ± 0·067

* ADH activity expressed in μmoles NAD reduced per minute ± S.D (Steel & Torrie, 1960) for four replicate determinations.

The ADH activity levels in the flooded roots of *Adh1* + /*Adh1* + , *Adh1* + /*Adh1-M9* and *Adh1-M9*/*Adh1-M9* plants were assayed (Table 2). As expected the activity level in flooded *Adh1-M9*/*Adh1-M9* roots is approximately 50 % of that in flooded *Adh1* + /*Adh1* + roots (cf. Harberd & Edwards, 1982). The ADH activity in flooded *Adh1* + /*Adh-M9* heterozygote roots is considerably lower than is observed in flooded *Adh1* + /*Adh1* + homozygote roots, the former being approximately 65 % of the latter. Hence whilst displaying an identical Group A : B : C activity ratio *Adh1* + /*Adh1-M9* flooded roots have an ADH activity level significantly lower than that observed in *Adh1* + /*Adh1* + flooded roots.

4. DISCUSSION

The results presented above suggest that ADH in barley is specified by three loci *Adh1*, *Adh2* and *Adh3* (Fig. 1), and not by two as was previously supposed (Harberd & Edwards, 1982). The single ADH band found in scutellum, embryo and endosperm of dry seed, and also in pollen (Harberd, 1981; Harberd & Edwards, 1982) is specified by the *Adh1* locus. This band is designated the Set I band. The existence of the six bands Sets I–VI in flooded *Adh1* + /*Adh1* + roots can be explained by the induction by flooding of the product of the *Adh1* locus and of the other two loci, *Adh2* and *Adh3*. ADH is a dimeric enzyme in a number of plants including barley (Fischer & Schwartz, 1973; Hart, 1971; Banuett-Bourrillon & Hague, 1979; Harberd & Edwards, 1982). The existence of the Set II band can be explained by the heterodimerization of the ADH1 and ADH3 polypeptides, the Set III band by heterodimerization of the ADH 1 and ADH2 polypeptides and

the Set V band by heterodimerization of the ADH2 and ADH3 polypeptides. The ADH activity in flooded *Adh1-M9/Adh1-M9* roots is contributed by Sets IV, V and VI only, suggesting that Sets I, II and III are abolished by a single mutation, a prediction of this model.

It is possible to propose alternative hypotheses which would explain the existence of the apparent multiple isozyme pattern observed in terms of post-translational modification. There is evidence that such hypotheses are unlikely to be correct. It has already been shown, by mutational analysis, that the ADH1 and ADH2 polypeptides cannot be related to one another by post-translational modification (Harberd & Edward, 1982). Similarly, the ADH3 polypeptide cannot be derived from ADH1 (or vice versa) since it clearly exists in *Adh1M9/Adh1-M9* homozygous flooded roots (Plate 1*a*). Hence, if post-translational modification or ligand binding are responsible for the existence of the ADH3 polypeptide they must be acting on the ADH2 polypeptide but not on the ADH1 polypeptide. It is unlikely that such processes could distinguish with total specificity between two polypeptides which probably differ only slightly in primary sequence and at higher levels of structure. Despite this argument, final proof that Sets IV and V contain a separately coded ADH3 polypeptide will only be obtained once an *Adh2* or *Adh3* null mutation has been isolated.

Investigations of the linkage relationships between the three loci using electrophoretic variants found in the wild barley, *Hordeum spontaneum* are currently in progress. Preliminary evidence suggests that *Adh1* and *Adh2* are indeed linked (as previously shown by Brown, 1980) but that *Adh3* is unlinked to the *Adh1–Adh2* gene complex.

Several possible causes of the deviation of the observed Group A : B : C ratio from that expected on the basis of equation (1) can be identified. Firstly, the ADH protomers in flooded roots might exist in two or more populations which do not intermix freely. This compartmentalization could be at the tissue, cellular or intracellular level. However, whilst considerable caution must be exercised when comparing the *in vitro* and *in vivo* behaviour of ADH monomers and protomers, the data from the *in vitro* dissociation and reassociation experiments suggest that the Group A : B : C ratio is a result of the properties of the ADH protomers themselves and not of sub-division of the ADH protomer pool. A second possibility is that the specific activity of the ADH monomers is inhibited in the heterodimers comprising the Group B isozymes, thus lowering the activity of the Group B isozymes but leaving Groups A and C unaffected. Thirdly, the Group A : B : C activity ratio could be accounted for if dimerization is non-random, and preferential homodimerization of the ADH protomers occurs. Further information on the Group A : B : C ratio will be obtained by determination of the underlying protein level ratio. It is of interest to note that no deviation from the binomial expectation for random dimerization with no inhibitive interaction between subunits has been reported for the maize ADHs.

As shown above (Table 2) the total ADH activity in *Adh1-M9/Adh1-M9* flooded roots is 55% of that in *Adh1+/Adh1+* flooded roots. Comparison of the ADH

activity in *Adh1+/Adh1-M9* flooded roots with the ADH activity in *Adh1+/Adh1+* flooded roots shows that the heterozygote contains considerably less than 100% of the activity in the homozygote but that the Group A : B : C ratio is constant between genotypes. Discussion of the significance of these findings is hindered by ignorance of the nature of the *Adh1-M9* mutation. It is not known if it is a structural or a regulatory mutation. However, it appears that *Adh1-M9* in heterozygous condition has uncovered a regulatory mechanism which maintains the constancy of the Group A : B : C ratio in flooded roots despite changes in the total ADH activity level.

It is interesting to speculate on the evolutionary relationships between the three *Adh* loci found in barley. Gene duplication and subsequent divergence is known to be a major source of the genetic variation which, through natural selection or genetic drift, results in evolutionary change (Ohno, 1970). It seems probable that the three *Adh* loci in barley are ancestrally related. It has also been suggested that gene duplications which cover tightly linked, *cis*-acting control elements will allow divergence of regulatory sequences whilst the ancestral sequences remain relatively conserved (Zuckerkandl, 1978). The *Adh* loci, which have very different patterns of expression with respect to tissue specificity, may well have evolved in this way.

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