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SOME PROPERTIES OF A HIGHER PLANT ALCOHOL DEHYDROGENASE

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Abstract—The alcohol dehydrogenase (EC 1.1.1.1) from germinating pea cotyledons has been purified approximately twenty-five fold. Preparations of the enzyme were found to be very unstable in the absence of sulphydryl reagents after chromatography on DEAE-Sephadex or after treatment with calcium phosphate gel. The enzyme was found to display broad specificity towards aliphatic alcohols but was specific for NAD and NADH. The Michaelis constants for ethanol and acetaldehyde were found to vary with pH and the reversibility characteristics of the reaction indicated that ethanol formation was favoured. On the basis of gel filtration studies, the molecular weight was found to be approximately 60,000. Enzyme activity was markedly inhibited by iodoacetate, iodoacetamide, *p*-chloromercuribenzoate, heavy metal ions and a number of chelating agents. The inhibition by iodoacetate and iodoacetamide was competitive with respect to ethanol concentration but non-competitive with respect to NAD concentration. Inhibition by *o*-phenanthroline was competitive with respect to NAD concentration and non-competitive with respect to ethanol concentration. It is concluded that the enzyme possesses certain catalytic properties in common with the alcohol dehydrogenases of horse-liver and yeast and also that the enzyme has importance in ethanol utilization in germinating tissues.

INTRODUCTION

THE GENERAL physical and catalytic properties of the alcohol dehydrogenases (alcohol: NAD^+ oxidoreductase, EC 1.1.1.1) from horse-liver and yeast are well known.[1] On the basis of these studies it is clear that catalysis involves the formation of ternary complexes with hydrogen being transferred directly without participation of the solvent. However, few detailed studies have been made of the enzyme from higher plant tissues. Cossins and Turner,[2] reporting on the alcohol dehydrogenase activity of pea cotyledons, showed that enzyme activity was inhibited by iodoacetate and that broad specificity existed for primary, secondary and tertiary aliphatic alcohols. Later papers[3,4] have confirmed these properties and recent studies of ethanol metabolism in germinating tissues have implicated this enzyme in the initial stages of ethanol utilization.[5,6]

In the present investigation, the preliminary studies of Cossins and Turner[2] have been extended using partially purified enzyme preparations in an attempt to further characterize this plant enzyme.

RESULTS

Changes in Enzyme Activity During Germination

In preliminary experiments samples of germinating seeds were examined for possible changes in the levels of alcohol dehydrogenase activity. Assays were performed on crude

[1] H. SUND and H. THEORELL, in *The Enzymes* (edited by P. D. BOYER, H. LARDY and K. MYRBÄCK), Vol. 7, p. 25. Academic Press, New York (1963).
[2] E. A. COSSINS and E. R. TURNER, *Ann. Botany* N.S. **26**, 591 (1962).
[3] Y. SUZUKI, *Phytochem.* **5**, 761 (1966).
[4] C. E. ERIKSSON, *Acta Chem. Scand.* **21**, 304 (1967).
[5] E. A. COSSINS and E. R. TURNER, *J. Exptl Botany* **14**, 290 (1963).
[6] D. S. CAMERON and E. A. COSSINS, *Biochem. J.* **105**, 323 (1967).

extracts of the cotyledons after removal of the developing embryos. The results are presented in Figs. 1 and 2. In agreement with earlier published work[7,8] the levels of enzyme activity rapidly rose to a maximum both on the basis of fresh weight of the tissue and protein content of the homogenates.

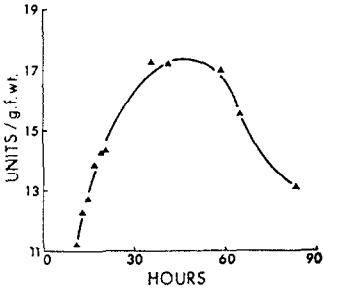

FIG. 1. CHANGES IN THE ALCOHOL DEHYDROGENASE CONTENT OF GERMINATING PEA COTYLEDONS.

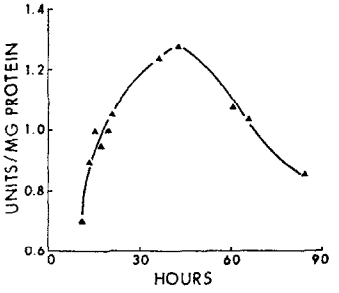

FIG. 2. CHANGES IN THE SPECIFIC ENZYME ACTIVITY OF HOMOGENATES DURING GERMINATION.

As 2-day-old cotyledons were found to contain maximal levels of alcohol dehydrogenase, tissue of this age was used as a source of the enzyme in all subsequent experiments. This maximal enzyme activity coincided with emergence of the radicles. The levels of enzyme activity during germination were not appreciably affected by previously soaking the seeds in 8×10^{-3} M chloramphenicol or 1×10^{-3} M *p*-fluorophenylalanine although these treatments resulted in marked inhibition of radicle growth. This indicates that such increases in enzyme activity are probably not related to enzyme synthesis but rather to activation of the enzyme. The exact nature of such activation remains to be determined.

Partial Purification and Stability of Enzyme Activity

A large number of procedures were examined in attempts to purify the alcohol dehydrogenase activity of crude homogenates. During such work it became clear that the enzyme was extremely unstable especially when subjected to dilution. For these reasons fractionation with $(NH_4)_2\ SO_4$ was found to be the most effective procedure. Protein precipitated in the range 50–55 per cent saturation with $(NH_4)_2\ SO_4$ was found to have a specific enzyme activity approximately twenty-five times greater than the original crude homogenate. The recovery of enzyme activity was approximately 20 per cent of that present in the crude tissue homogenate. Attempts to purify the enzyme further by means of calcium phosphate gel treatment

[7] J. GOKSÖYR, E. BOERI and R. K. BONNICHSEN, *Acta Chem. Scand.* **7**, 657 (1953).
[8] A. MAFFEI FACCIOLO, *Boll. Soc. Ital. Biol. Sper.* **35**, 2166 (1959).

followed by column chromatography on DEAE-cellulose, DEAE-Sephadex or hydroxylapatite resulted in poor recoveries of enzyme activity. Also attempts to fractionate the protein by heat treatment, acetone and ethanol precipitation were unsuccessful. For these reasons all subsequent experiments were conducted using the enzyme after $(NH_4)_2$ SO_4 fractionation.

The instability of the enzyme after various purification treatments is illustrated in Fig. 3. Loss of enzyme activity could be partially reduced by addition of 2-mercaptoethanol or dithiothreitol (Fig. 3C) but was pronounced even in the presence of 2-mercaptoethanol when the enzyme was eluted from DEAE-Sephadex. Inactivation also occurred when the 50–55 per cent $(NH_4)_2SO_4$ fraction was dissolved in 0·1 M potassium phosphate buffer (pH 8·5) containing 0·01 M mercaptoethanol and stored at −20° for 24 hr. Clearly this plant alcohol dehydrogenase is considerably more unstable than the corresponding dehydrogenases from mammalian liver and yeast. The partial protection afforded by sulphydryl reagents does however suggest that this enzyme, like other well-characterized alcohol dehydrogenases, contains free sulphydryl groups essential for enzyme activity. Free sulphydryl groups could in fact be readily determined by measuring mercaptide formation in the presence of *p*-chloromercuribenzoate.[9] Also enzyme activity was readily lost on aerating enzyme preparations in the absence of 2-mercaptoethanol.

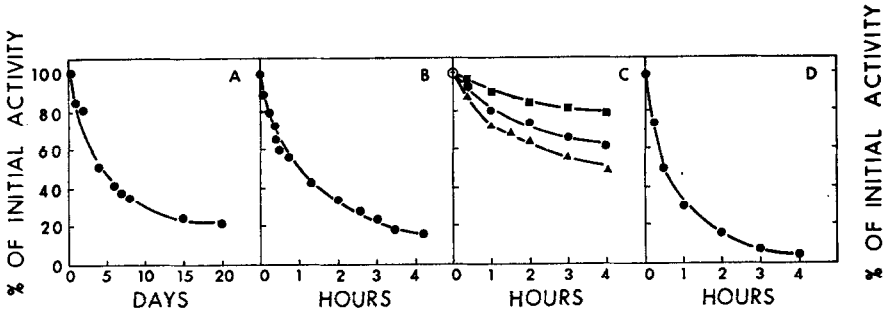

FIG. 3. STABILITY OF ALCOHOL DEHYDROGENASE AFTER VARIOUS TREATMENTS.
A, crude homogenate. B, 50–55 per cent $(NH_4)_2SO_4$ fraction treated with calcium phosphate gel. C, 50–55 per cent $(NH_4)_2SO_4$ fraction, ▲—▲ alone; ●—● with addition of 0·01 M dithiothreitol; ■—■ with addition of 0·01 M 2-mercaptoethanol. D, after chromatography of 50–55 per cent $(NH_4)_2SO_4$ fraction on DEAE-Sephadex in the presence of 0·01 M 2-mercaptoethanol.

Estimation of Molecular Weight by Gel Filtration

Samples of the crude homogenate and the 50–55 per cent $(NH_4)_2SO_4$ fraction containing less than 5 mg protein were passed through columns of Sephadex G-100 together with highly purified proteins of known molecular weight. The column effluent was assayed for alcohol dehydrogenase activity and for protein as described in the Experimental section. Elution volumes were estimated as described by Andrews[10] and plotted against log molecular weight for the proteins applied to the column (Fig. 4). On the basis of four separate experiments the average molecular weight for plant alcohol dehydrogenase was estimated to be approximately 60,000. In elutions which included samples of crystalline yeast and horse-liver alcohol dehydrogenases, elution volumes from Sephadex G-100 and G-200 were obtained which were distinct from that shown by the plant enzyme but which were in agreement with molecular weights of approximately 150,000 and 80,000 respectively.

[9] P. D. BOYER, *J. Am. Chem. Soc.* **76**, 4331 (1954).
[10] P. ANDREWS, *Biochem. J.* **91**, 222 (1964).

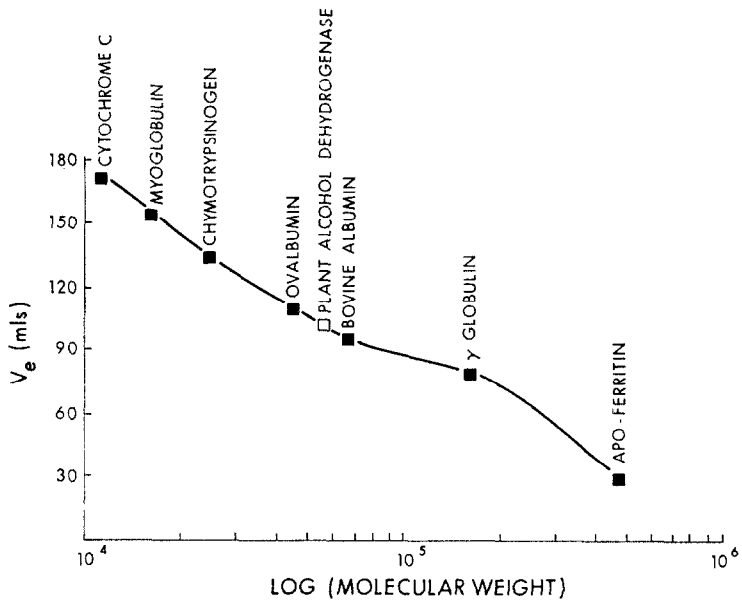

FIG. 4. GEL FILTRATION OF ALCOHOL DEHYDROGENASE ON SEPHADEX G-100.

Purified proteins (approx. 2-mg samples) were applied to columns of Sephadex G-100 and eluted as described by Andrews.[10] Apo-ferritin (mol. wt. 480,000) had an elution volume corresponding to the void volume of the columns.

General Catalytic Properties of the Enzyme

Enzyme activity was markedly effected by pH as shown in Figs. 5 and 6. The oxidation of ethanol occurred at maximal rates at pH 8·8. In contrast the reduction of acetaldehyde occurred most rapidly at pH 6·3. Similar differences in the pH optima for the oxidation and reduction reactions have been reported for the horse[11] and human liver[12] enzymes. The oxidation of ethanol (5×10^{-2} M) was found to be readily reversible at pH 8·8 by addition of $1{\cdot}7 \times 10^{-3}$ M acetaldehyde. However, the reduction of acetaldehyde ($3{\cdot}4 \times 10^{-3}$ M) at pH 8·8 was not reversed by addition of 1×10^{-1} M ethanol. It is clear that the enzyme has

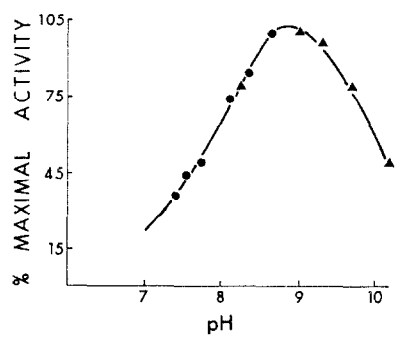

FIG. 5. THE EFFECT OF pH ON THE INITIAL REACTION VELOCITIES OF ETHANOL OXIDATION.

●—●, 0·05 M KH_2PO_4 buffer; ▲—▲, 0·05 M K_2HPO_4 buffer.

[11] H. THEORELL and R. K. BONNICHSEN, *Acta Chem. Scand.* **5**, 1105 (1951).
[12] J-P VON WARTBURG, J. L. BETHUNE and B. L. VALLEE, *Biochemistry* **3**, 1775 (1964).

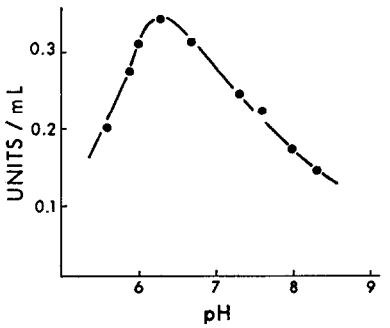

FIG. 6. THE EFFECT OF pH ON THE INITIAL REACTION VELOCITIES OF ACETALDEHYDE REDUCTION. 0·05 M KH_2PO_4 and K_2HPO_4 buffers were used.

considerably greater affinity for acetaldehyde at this pH value. Like several other alcohol dehydrogenases,[1] the enzyme was found to be fairly specific for NAD and NADH. Enzyme activity in the presence of NADP was approximately 1 per cent of that observed in the presence of NAD. In agreement with other published work[2, 4] the enzyme was not however highly specific for ethanol (Table 1) as NAD reduction was observed in the presence of several aliphatic alcohols. The broad specificity of this enzyme for aliphatic alcohols is also characteristic of this enzyme from other sources.[1]

TABLE 1. ENZYME ACTIVITY IN THE PRESENCE OF VARIOUS ALCOHOLS

Alcohol	Final concentration (mM)	Relative enzyme activity
Ethanol	50	100
Methanol	50	0·406
n-Propanol	50	36·04
n-Butanol	Sat. aqueous soln.	24·36
Isopropanol	50	0·406
Cyclopentanol	Sat. aqueous soln.	0·406
Cyclohexanol	Sat. aqueous soln.	0
Glycerol	50	0

The intracellular distribution of alcohol dehydrogenase activity is shown in Table 2. The cytoplasmic fraction was found to have both the highest specific enzyme activity and total enzyme activity. In agreement with similar studies conducted with etiolated pea

TABLE 2. THE INTRACELLULAR DISTRIBUTION OF ALCOHOL DEHYDROGENASE

Fraction	Total units	Units/mg protein
Whole homogenate*	2640	0·80
Mitochondrial	10	0·017
Microsomal	17	0·19
Cytoplasmic	1085	0·92

* Derived from 100 g of 2-day-old cotyledons.

stems,[13, 14] the mitochondrial fraction was found to contain measurable levels of alcohol dehydrogenase activity.

In kinetic experiments the Michaelis constants for ethanol and acetaldehyde were determined at various pH values (Table 3). It is clear that the enzyme displayed greatest affinity for these substrates at pH 7·3. The Michaelis constants for NAD and NADH at pH 8·5 were both found to be $6{\cdot}6 \times 10^{-5}$ M.

TABLE 3. MICHAELIS CONSTANTS* FOR ETHANOL AND ACETALDEHYDE

pH	K_m (ethanol)	K_m (acetaldehyde)
5·3		$2{\cdot}0 \times 10^{-3}$ M
6·2	$2{\cdot}4 \times 10^{-2}$ M	$1{\cdot}6 \times 10^{-3}$ M
7·3	$1{\cdot}6 \times 10^{-3}$ M	$1{\cdot}2 \times 10^{-3}$ M
8·3	$3{\cdot}3 \times 10^{-2}$ M	$5{\cdot}0 \times 10^{-3}$ M

* Determined by the method of Lineweaver and Burk.[15]

The Effects of Sulphydryl Group Reagents

In order to determine whether the protection afforded by 2-mercaptoethanol and dithiothreitol (Fig. 3C) was due to a requirement for free sulphydryl groups, further experiments were conducted with various sulphydryl group reagents. Enzyme activity was markedly inhibited in the presence of iodoacetate, iodoacetamide and *p*-chloromercuribenzoate. The degree of inhibition was in all cases dependent on the concentration of the inhibitor and was increased by pre-incubating the enzyme with the inhibitor before initiating the reaction. Inhibition by iodoacetate was found to be competitive with respect to ethanol concentration and non-competitive with respect to NAD concentration. Similar results were obtained for iodoacetamide inhibition. Enzyme activity was also progressively inhibited by zinc and cobaltous ions. Complete inhibition of enzyme activity (1 mg protein) was observed when these ions were present at concentrations of $1{\cdot}5 \times 10^{-3}$ M and $1{\cdot}6 \times 10^{-3}$ M respectively.

TABLE 4. PARTIAL PROTECTION AGAINST IODOACETATE INHIBITION BY NAD

Pre-incubation for 2 min at 23°	Subsequent additions	V_i/V_c*	
		Experiment 1	Experiment 2
Enzyme+buffer	NAD+ethanol	1·0	1·0
Enzyme+buffer	Inhibitor+NAD+ethanol	0·44	0·44
Enzyme+buffer	NAD+inhibitor+ethanol	0·64	0·66
Enzyme+buffer+NAD	Inhibitor+ethanol	0·64	0·66

Note. The enzyme (28 μg protein) was pre-incubated with 0·5 ml of 0·1 M phosphate buffer (pH 8·5). Additions of iodoacetate, 1 μmole; NAD, 1·36 μmoles in experiment 1, 0·34 μmole in experiment 2; and ethanol, 150 μmoles were made as indicated. Total volume 3 ml.

* V_i = initial reaction velocity in the presence of the inhibitor.
V_c = initial reaction velocity in the controls.

[13] D. D. DAVIES, *J. Exptl Botany* **7**, 203 (1956).
[14] T. E. RAGLAND and D. P. HACKETT, *Arch. Biochem. Biophys.* **108**, 479 (1964).
[15] H. LINEWEAVER and D. BURK, *J. Am. Chem. Soc.* **56**, 658 (1934).

These data show that free sulphydryl groups are essential for enzyme activity. Furthermore the competition between these reagents and ethanol points to such groups as having importance in substrate binding. The sulphydryl groups may also have a role in coenzyme binding as partial protection against iodoacetate inhibition was obtained by prior additions of NAD (Table 4). This protection was obvious when the enzyme was pre-incubated with NAD or when the subsequent addition of NAD was made before addition of the inhibitor. Furthermore, additions of ethanol and NAD resulted in marked decreases in the free sulphydryl groups as measured spectrophotometrically with *p*-chloromercuribenzoate. The importance of free sulphydryl groups as binding sites for the substrates and coenzymes of other alcohol dehydrogenases is well documented[1] and the present work indicates a similar role in the plant enzyme.

The Effects of Chelating Agents

Enzyme activity was readily inhibited by a number of well-known chelating agents. The final concentrations of these inhibitors giving 50 per cent inhibition were, L-thyroxine, 1×10^{-2} M; 8-hydroxyquinoline, 5×10^{-4} M; EDTA, 6×10^{-2} M; sodium sulphide and α,α^1-dipyridyl, $1{\cdot}5 \times 10^{-1}$ M and sodium azide, 4×10^{-1} M. *o*-Phenanthroline was also found to be an effective inhibitor of enzyme activity. The degree of inhibition was affected by inhibitor concentration but was not affected by preincubating the enzyme with the inhibitor prior to initiating the reaction. In kinetic experiments, the inhibition caused by *o*-phenanthroline was examined in relation to NAD and ethanol concentration. The inhibition was found to be competitive with respect to NAD concentration and non-competitive with respect to ethanol concentration. On the basis of these data it is conceivable that the enzyme contains a metal component essential for activity and further that such a metal may have importance in coenzyme binding. It is of interest to note in this connexion that in preliminary studies considerable quantities (approximately 10 μg/mg protein) of bound zinc were detected in thoroughly dialyzed enzyme preparations by atomic absorption spectrophotometry. However, highly purified preparations of the enzyme will have to be examined before the identity of the metal component can be established unequivocally.

DISCUSSION

From the present and earlier investigations,[2–4] it is clear that the alcohol dehydrogenase from germinating pea cotyledons should be included in the EC 1.1.1.1 category. In common with mammalian and yeast alcohol dehydrogenases, the plant enzyme possesses free sulphydryl groups and is inhibited by chelating agents. The major characteristics of these enzymes are summarized in Table 5.

Regarding substrate specificity, the plant enzyme resembles the yeast enzyme with the exception that tertiary alcohols are not attacked by the latter enzyme.[1] Also, the plant enzyme resembles the enzyme from yeast in its coenzyme requirements. It is interesting to note in this connexion that Stafford and Vennesland[16] have shown that the alcohol dehydrogenase of wheat germ, like the enzyme from horse-liver, can utilize NAD or NADP in the oxidation of ethanol. The three enzymes differ markedly in molecular weight (Table 5). Although the value of 60,000 for the plant enzyme is based entirely on gel filtration studies it is likely that other techniques will corroborate these findings as samples of the horse-liver and yeast enzymes gave elution volumes corresponding to within ± 10 per cent of their known

[16] H. A. STAFFORD and B. VENNESLAND, *Arch. Biochem. Biophys.* **44**, 404 (1953).

molecular weights. The values of the Michaelis constants vary considerably (Table 5) but all of the enzymes have generally more affinity for acetaldehyde even at pH values where ethanol oxidation is optimal.[1]

TABLE 5. PHYSICAL AND CATALYTIC PROPERTIES OF MAMMALIAN, YEAST AND PLANT ALCOHOL DEHYDROGENASES

	Horse-liver ADH*	Yeast ADH*	Plant ADH
Alcohol	n; sec; tert; aliphatic aromatic (excluding methanol)	n; sec; aliphatic (including methanol)	n; sec; tert; aliphatic (including methanol)
Coenzyme	NAD; NAD analogs also NADP	NAD; NAD analogs not NADP	NAD and NADP
Mol. Wt.	84,000	150,000	*ca.* 60,000
K_m values (M)			
Ethanol	$2{\cdot}4 \times 10^{-3}$ (pH 9·5)	$1{\cdot}4 \times 10^{-2}$ (pH 8·2)	$3{\cdot}3 \times 10^{-2}$ (pH 8·3)
Acetaldehyde	$2{\cdot}1 \times 10^{-4}$ (pH 6·95)	$2{\cdot}5 \times 10^{-4}$ (pH 7·15)	$1{\cdot}2 \times 10^{-3}$ (pH 7·3)
NAD	$5{\cdot}0 \times 10^{-6}$ (pH 8·0)	$1{\cdot}7 \times 10^{-4}$ (pH 7·9)†	$6{\cdot}6 \times 10^{-5}$ (pH 8·5)
NADH	$5{\cdot}4 \times 10^{-6}$ (pH 8·0)	$2{\cdot}3 \times 10^{-5}$ (pH 7·9)†	$6{\cdot}6 \times 10^{-5}$ (pH 8·5)
–SH groups	Essential	Essential	Essential
Chelators	Inhibitory	Inhibitory	Inhibitory

* Data from Sund and Theorell.[1]
† Data from Hayes and Velick.[17]

Considering the high affinity displayed by the plant enzyme for acetaldehyde and the reversibility characteristics of this enzyme, it is possible that unless acetaldehyde is rapidly removed by oxidative reactions, ethanol accumulation will occur in the cotyledons. Conditions favourable for ethanol accumulation appear to prevail during the early stages of germination.[5, 18] As aerobic respiration becomes established in such tissues, the accumulated ethanol will be readily metabolized via the intermediary formation of acetaldehyde[5] by an apparent reversal of alcohol dehydrogenase. This reversal is probably dependent on the availability of NAD and on the affinity for acetaldehyde of enzymes involved in subsequent oxidative reactions. Recent studies by Effer and Ranson[19] have shown marked increases in the NAD/NADH ratio after seedlings are transferred from anaerobic to aerobic conditions. If such changes occur in pea cotyledons, ethanol oxidation would be favoured. Also, recent studies by Oppenheim and Castelfranco[20] have emphasized the importance of acetaldehyde dehydrogenase during the utilization of ethanol by seedlings. The high affinity of this enzyme for acetaldehyde would tend to maintain low levels of this compound in the tissues and therefore contribute to the depletion of ethanol content that occurs during germination.

EXPERIMENTAL

Chemicals

All chemicals used in this investigation were purchased from Fisher Scientific Company, Nutritional Biochemicals Corporation, Mann Research Laboratories and Sigma Chemical Company, and were of the highest purity commercially available. All solutions were prepared using de-ionized glass re-distilled water.

[17] J. E. HAYES and S. F. VELICK, *J. Biol. Chem.* **207**, 225 (1954).
[18] E. A. COSSINS and E. R. TURNER, *Nature Lond.* **183**, 1599 (1959).
[19] W. R. EFFER and S. L. RANSON, *Plant Physiol.* **42**, 1053 (1967).
[20] A. OPPENHEIM and P. A. CASTELFRANCO, *Plant Physiol.* **42**, 125 (1967).

Plant Material

Seeds of pea (*Pisum sativum* L. variety Homesteader) were soaked in distilled water for 18 hr at 25° in darkness. They were then germinated in moist vermiculite for 2 days at 28°.

Partial Purification of Alcohol Dehydrogenase Activity

After removal of the testas and embryos, the cotyledons (100 g) were ground in 150 ml of 0·1 M K phosphate buffer (pH 8·5) containing 0·01 M 2-mercaptoethanol at 2° using a Waring blendor. All subsequent operations were carried out at 4°. The crude homogenate was passed through fine cheesecloth and immediately centrifuged at 16,000 × *g* for 20 min to remove cellular debris. The supernatant was adjusted to 50 per cent saturation by the slow addition of sat. $(NH_4)_2SO_4$. After removal of the precipitated protein by centrifugation, more sat. $(NH_4)_2SO_4$ was added to achieve 55 per cent saturation. The protein precipitated was then collected by a further centrifugation and dissolved in 50 ml of 0·01 M K phosphate buffer (pH 8·5) containing 0·01 M 2-mercaptoethanol. The enzyme solution so obtained was then dialyzed against the same buffer for 10 hr at 2°.

An aliquot containing approximately 14 mg of protein was applied to a 15 × 1 cm column of DEAE-Sephadex (A-50, coarse mesh) which had previously been equilibrated with 0·01 M K phosphate buffer (pH 8·5) containing 0·01 M 2-mercaptoethanol. Enzyme activity was retained by the column and was subsequently eluted using a gradient of phosphate buffer at pH 8·5. The mixing vessel contained initially 200 ml of 0·01 M K phosphate buffer (pH 8·5) containing 0·01 M 2-mercaptoethanol. The reservoir contained 0·4 M K phosphate buffer (pH 8·5) containing 0·01 M 2-mercaptoethanol. Fractions of 5 ml were collected every 3 min. Fractions 15–24 contained appreciable levels of enzyme activity. In some experiments the protein obtained from $(NH_4)_2SO_4$ fractionation (50–55 per cent saturation) was treated with calcium phosphate gel as follows. Prior to gel treatment the precipitated protein (approximately 3·0 mg) was dissolved in 10 ml of 0·1 M K phosphate buffer (pH 8·5) containing 0·01 M 2-mercaptoethanol and dialyzed against the same buffer for 4 hr at 2°. Calcium phosphate gel was prepared according to the method of Keilin and Hartree.[21] The gel was aged for 12 months at 2° prior to use. Using a protein:gel ratio of 0·003, enzyme activity was readily absorbed by the gel. The gel was then washed with 3 ml of 0·1 M K phosphate buffer (pH 8·5) and the supernatant discarded. Enzyme activity was recovered from the gel by a further wash of 0·2 M K phosphate buffer (pH 8·5). Protein was assayed colorimetrically.[22]

Assay of Enzyme Activity

Alcohol dehydrogenase activity was assayed spectrophotometrically as described by Racker.[23] Increases in extinction at 340 nm due to the production of NADH were followed using a Beckman double-beam spectrophotometer equipped with a potentiometric recorder. Initial reaction velocities were calculated from the tangent drawn to the reaction time course curve. The standard reaction system contained in a final volume of 3 ml, ethanol, 150 μmoles; NAD, 0·68 μmoles; K phosphate buffer (pH 8·5), 50 μmoles, and approximately 0·06 units of enzyme. The reaction was carried out at 25° and initiated by addition of ethanol. One unit of enzyme activity is defined as the amount catalyzing the production or consumption of 1 μmole of NAD/min under the defined experimental conditions.

Estimation of Molecular Weight by Gel Filtration

Gel filtration of enzyme preparations was carried out using Sephadex G-100 and G-200 (Pharmacia, Uppsala, Sweden) by the method described by Andrews.[10,24] Samples of highly purified proteins (Mann Research Laboratories) were used to obtain standard elution volumes. Crystalline preparations of horse-liver and yeast alcohol dehydrogenases were purchased from Sigma Chemical Company. The positions of these proteins in the effluent from the column were determined on the basis of enzyme activity or absorbance where applicable.[10]

Fractionation of Extracts by Centrifugation

Samples of 2-day-old cotyledons (100 g) were ground in a mortar with 100 ml of sucrose–phosphate buffer (0·5 M sucrose – 0·1 sodium phosphate, pH 8·5) at 4°. Cellular debris was removed by passing the homogenate through fine cheese-cloth and by a subsequent centrifugation at 2000 × *g* for 15 min. Differential centrifugation of this supernatant was then carried out to yield mitochondrial (23,000 × *g* for 30 min), microsomal (54,000 × *g* for 60 min) and cytoplasmic fractions. The mitochondrial fraction was resuspended in 30 ml of sucrose–phosphate buffer and then recentrifuged. In order to concentrate the enzyme activity associated

[21] D. KEILIN and E. F. HARTREE, *Proc. R. Soc. Lond. B.* **8**, **124**, 397 (1938).
[22] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[23] E. RACKER, *J. Biol. Chem.* **184**, 313 (1950).
[24] P. ANDREWS, *Biochem. J.* **96**, 595 (1965).

with these fractions, additions of saturated ammonium sulphate (up to 60 per cent saturation) were made and the precipitated protein dissolved in 10 ml of Na phosphate buffer (pH 8·5) before enzyme assay.

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