Phytochemistry, 1966, Vol. 5, pp. 1147 to 1152. Pergamon Press Ltd. Printed in England

# ISOENZYMES OF MALATE DEHYDROGENASE FROM BARLEY SEEDLINGS

S. B. YUE*

Department of Botany, The University, Bristol 8

(*Received* 26 *January* 1966)

**Abstract**—A partial purification of malate dehydrogenase from barley seedlings has been achieved. By ion-exchange chromatography the enzymic activity could be separated into two distinct peaks, suggesting that both anionic and cationic forms exist. Differential centrifugation indicates that the two forms are separated spatially within the cell, one being associated with the supernatant and the other with a particulate fraction. Further evidence for two isoenzymes was obtained from their different apparent $K_m$ values for L-malate and NAD.

## INTRODUCTION

EARLIER studies on the malate dehydrogenase (1.1.1.37:L-Malate:NAD oxidoreductase) of higher plants have left some doubt whether the enzyme exists as two isoenzymes. Thus Price and Thimann[1] demonstrated that the dehydrogenase was apparently associated with both mitochondrial and supernatant fractions, but were uncertain as to the extent their results might be due to adsorption of the supernatant enzyme upon the particulate fraction. More recently Davies[2] has shown that by chromatography on diethylaminoethylcellulose (DEAE-cellulose) the enzyme can be resolved into two fractions, but concluded from determinations of their affinity for oxalacetate that they were not significantly different. In the present work the enzyme of young barley seedlings (*Hordeum distichon*) has been investigated and found to exist in two forms, chromatographically and kinetically distinct, one associated with a particulate fraction (mitochondria?) and one with the supernatant. This situation closely parallels that in animal tissues where it has been clearly established that two isoenzymes exist.

## RESULTS AND DISCUSSION

A partial purification of malate dehydrogenase (MDH) can be obtained by treating a homogenate of barley seedlings with ammonium sulphate, and collecting the fraction between 34 and 44% saturation (Table 1). When this preparation is chromatographed on DEAE-cellulose, two peaks (A and B) of MDH activity can be distinguished (Fig. 1), representing 25- and 46-fold purification respectively. Similar elution patterns have been reported for the enzyme from cauliflowers[2] and rat liver;[3] in the latter case the faster-running fraction was shown to be associated with the mitochondria, while the second was derived from the supernatant after centrifugation.

* Present address: Department of Botany, University of Hong Kong, Hong Kong.

[1] C. A. PRICE and K. V. THIMANN, *Plant Physiol.* **29**, 113 (1954).
[2] D. D. DAVIES, *Biochem. J.* **80**, 93 (1961).
[3] C. J. R. THORNE, *Biochem. Biophys. Acta* **42**, 175 (1960).

TABLE 1. PURIFICATION OF MDH FROM YOUNG BARLEY EMBRYOS

| Fraction | Total activity[a] | Specific activity[b] | Recovery[c] (%) | Purification[c] factor |
|---|---|---|---|---|
| Crude homogenate | 2058 | 4 | 100 | 1 |
| 33–44% saturation $(NH_4)_2SO_4$ ppt. | 1667 | 19 | 81 | 5 |
| DEAE-cellulose eluate (Peak A) | 259 | 94 | 13 | 25 |
| DEAE-cellulose eluate (Peak B) | 524 | 169 | 26 | 46 |

[a] $\Delta E_{340\,m\mu}$ of 0·001/min. [b] $\Delta E_{340\,m\mu}$ of 0·001/min/mg. [c] Relative to crude homogenate fraction.

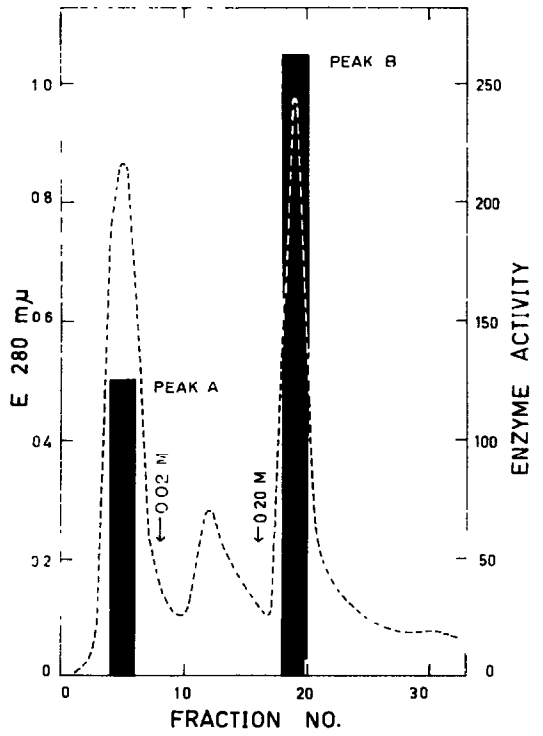


FIG. 1. COLUMN CHROMATOGRAPHY OF PARTIALLY PURIFIED MDH FROM BARLEY EMBRYOS ON DEAE-CELLULOSE COLUMN.

Enzyme preparation contains 6 mg ammonium sulphate-precipitated protein. Column (8 × 1·6 cm) developed by stepwise increases of phosphate buffer concentration (pH 8·0) as indicated. A unit of activity is expressed as the amount of enzyme producing an extinction change at 340 mμ of 0·001/min. Dashed line indicates protein concentration of fractions as measured at 280 mμ. Shaded areas represent MDH activity.

Centrifugation studies on homogenates of barley embryos suggest that MDH is present in both particulate and supernatant fractions. The results of two such experiments are presented in Table 2. These experiments differed in the concentration of phosphate used in the extraction medium; in experiment I 0·2 M phosphate was used, while in experiment II

the concentration was only 0·002 M. In both experiments activity was chiefly associated with the 5000 *g* and supernatant fractions, but the proportion in the 5000 *g* fraction (hereafter designated as mitochondrial fraction) was greatest in experiment I; this is consistent with the increased tendency for mitochondria to break down at low phosphate concentrations.[4] Even in experiment I it is probable that some mitochondrial breakdown has occurred and the supernatant may therefore be of mixed origin, but unless adsorption of supernatant enzyme onto the mitochondria has also occurred the particulate fraction should contain malate dehydrogenase deriving only from mitochondria.

TABLE 2. INTRACELLULAR DISTRIBUTION OF MDH FROM YOUNG BARLEY EMBRYOS

| Subcellular fraction ($g \times 10^3$) | Specific activity | | Total activity | | Total activity (%) | |
|---|---|---|---|---|---|---|
| | I | II | I | II | I | II |
| 1 (5 min) | 660 | 420 | 2,880 | 1,800 | 2·4 | 1·0 |
| 3·6 (12 min) | 955 | 1,050 | 6,720 | 7,440 | 5·5 | 4·2 |
| 5 (15 min) | 1,640 | 925 | 18,600 | 9,600 | 15·3 | 5·4 |
| 40 (15 min) | 231 | 326 | 2,720 | 5,500 | 2·2 | 3·1 |
| 75 (90 min) | 85 | 73 | 1,280 | 1,080 | 1·1 | 0·6 |
| Supernatant | 1,560 | 3,640 | 89,400 | 152,000 | 73·5 | 85·6 |

The extraction media used in Experiments I and II were 0·4 M sucrose in 0·2 M phosphate buffer (pH 6·4) and 0·4 M sucrose in 0·002 M phosphate buffer (pH 6·5) respectively.

Total and specific activities are expressed as in Table 1. 66 ml of cell-free homogenate were used for centrifugation in both Experiments I and II.

For chromatographic studies the material of experiment II was used, rather than that of experiment I, because the lower phosphate level interfered less with enzyme adsorption on DEAE-cellulose. The results are shown in Fig. 2. The general elution pattern is similar to that shown in Fig. 1, but the previous treatment of the enzyme preparations apparently alters somewhat their chromatographic behaviour, since much higher buffer concentrations are here needed for elution than were required for the ammonium sulphate-precipitated enzyme. The mitochondrial fraction contains substantially only one type of MDH, 93 per cent of the total activity being found in peak A. However, the activity of the supernatant fraction was resolved evenly into two peaks (A′ and B′); peak A′ corresponded in elution position with peak A in the mitochondrial fraction. From these data it may be inferred that the activity in peak B′ represents the supernatant enzyme; that in peak B of the mitochondrial fraction being in fact contamination from the supernatant retained in the pellets after decantation. It then follows that the activity in both peaks A and A′ represents the mitochondrial enzyme. If this is the case, it must be postulated that the majority of the mitochondrial enzyme was leached into the soluble phase, and this further supplements the earlier observation that phosphate concentration is important in the maintenance of the mitochondrial integrity. This distribution of activity is consistent with the data presented in Table 2. There is thus firm evidence for two malate dehydrogenases in barley, differing in their chromatographic behaviour and associated with different parts of the cell.

[4] A. MILLERD, *Arch. Biochem. Biophys.* **42**, 149 (1953).

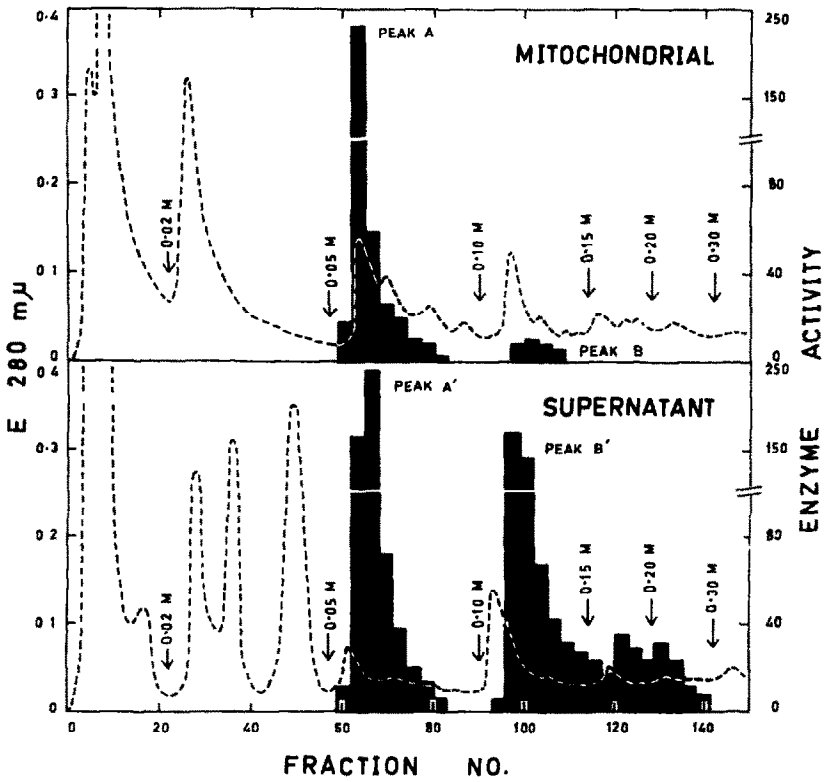


FIG. 2. COLUMN CHROMATOGRAPHY OF BARLEY MITOCHONDRIAL AND SUPERNATANT MDH'S ON DEAE-CELLULOSE COLUMN.

Mitochondrial and supernatant fractions equivalent to 14·6 g and 2·5 g fresh weight of tissue respectively. Column (19·5 × 1·0 cm) developed by stepwise increases of phosphate buffer (pH 8·0) as indicated. Unit of activity and symbols as in Fig. 1.

When Davies[2] examined his preparations from cauliflower he was unable to detect significant differences in the Michaelis constant ($K_m$) for oxalacetate between his two fractions. In the present study the constants for malate and nicotinamide-adenine dinucleotide (NAD) have been investigated instead, and here the evidence is much more clear-cut. As can be seen from Table 3, the peak A′ and peak B′ enzymes of the supernatant differ markedly in their Michaelis constants, but the $K_m$'s for peak A enzyme are practically the same as those for peak A′. This further confirms the conclusion that the supernatant peak A′ material is the result of mitochondrial breakdown, as well as emphasizing the differences between the two malate dehydrogenase isoenzymes.

There is at present little indication as to why plants and animals have two malate dehydrogenases and what, if any, separate function each may have. The supernatant enzyme from animals is reported[5] to be closely associated with glutamate-oxalacetate transaminase and it is noteworthy that the present author[6] has found the same to be true in plants; a fuller account of this will be published in a later paper. These observations suggest that the pair of enzymes may have some special function in cells, perhaps in connexion with glutamate breakdown or with aspartate synthesis.

[5] H. LIS and P. FASELLA, *Biochim. Biophys. Acta* 33, 567 (1959).
[6] S. B. YUE, Ph.D. Thesis, University of Bristol (1963).

TABLE 3. COMPARISON OF APPARENT $K_m$ VALUES FOR L-MALATE AND NAD OF PARTIALLY PURIFIED SUPERNATANT AND MITOCHONDRIAL MDH'S

| Cellular fraction | $K_m$ values | |
|---|---|---|
| | L-malate ($\times 10^{-3}$ M) | NAD ($\times 10^{-4}$ M) |
| Mitochondrial, Peak A | 50·0 | 17·5 |
| Supernatant, Peak A′ | 39·3 | 17·1 |
| Supernatant, Peak B′ | 8·2 | 3·4 |

Rates of oxidation of L-malate (in the range of $1{\cdot}79 \times 10^{-3}$ to $1{\cdot}96 \times 10^{-2}$ M) were measured in 0·2 M phosphate buffer, pH 8·3, in the presence of $1{\cdot}07 \times 10^{-3}$ M NAD at laboratory temperature. Rates of reduction of NAD (in the range of $2{\cdot}14 \times 10^{-4}$ to $5{\cdot}36 \times 10^{-4}$ M) were measured in the same buffer in the presence of $7{\cdot}14 \times 10^{-3}$ M L-malate.

$K_m$ values were obtained by extrapolation of Lineweaver and Burk[7] plots.

## EXPERIMENTAL

### *Material*

Barley (*Hordeum distichon* var. Spratt Archer) was used throughout the investigation. Grains were sterilized, by soaking in a 2% (w/v) solution of calcium hypochlorite for 2 hr, and germinated in pyrex dishes, lined with moist blotting paper, in a thermostatically controlled room at $24 \pm 1°$. The seedlings were harvested after 60–65 hr and dissected into endosperms and embryos. The dissected embryos were kept at 5° until use.

### *Preparation and Partial Purification of Enzyme*

The pre-chilled embryos were ground in a cold mortar with an equal weight of fine acid-washed sand and ice-cold 0·2 M phosphate buffer at pH 8·3 (1 ml/g fresh weight of tissue). The homogenate was strained through "Terylene" gauze, pH adjusted to 8·3 with 1N-NaOH, and centrifuged at 25,000 *g* at 5° for 10 min. To the supernatant was added solid ammonium sulphate at 5° to give 34% saturation. After 30 min the precipitated protein was removed by centrifugation. The ammonium sulphate concentration of the supernatant was then increased to 44% saturation After another 30 min at 5° the precipitated protein was collected by centrifugation, dissolved in 0·002 M phosphate buffer (pH 8·0) and assayed for activity. The enzyme was routinely stored in condition of 44% saturation with ammonium sulphate at −15° for as long as 3 months without appreciable loss of activity.

### *Column Chromatography on DEAE-cellulose*

The cellulose was equilibrated in 0·002 M phosphate buffer (pH 8·0) and was treated in a Waring Blendor to attain homogeneity. Small particles were removed by flotation and the rest of the cellulose was poured as a slurry into a column. The enzyme, in solution in the same buffer, was applied and the column was washed with more buffer to remove unadsorbed and loosely adsorbed materials. Elution was accomplished by stepwise increases in the concentration of buffer. Chromatographic fractionations were carried out at laboratory temperature, employing a flow rate of about 70 ml per hour under air pressure, 3·2 ml fractions being collected by means of an automatic fraction collector.

[7] H. LINEWEAVER and D. BURK, *J. Am. Chem. Soc.* **56**, 658 (1934).

*Differential Centrifugation*

A cell-free homogenate of barley embryos was prepared by grinding the tissues in a chilled mortar, without the addition of sand as an abrasive, and by centrifugation at 400 *g* for 1 min. The dispersion medium employed was 0·4 M sucrose buffered at pH 6·5 (3 ml/g fresh weight).

Fractionation of subcellular components was accomplished by differential centrifugation, employing forces of 1000 *g* for 5 min, 3600 *g* for 12 min, 5000 *g* for 15 min, 40,000 *g* for 15 min and 75,000 *g* for 90 min. No attempt was made in identifying the particles with the cytoplasmic components. The particulate fraction were suspended and homogenized in 0·002 M phosphate buffer (pH 8·3) without prior washing by resuspension in the extracting medium and recentrifugation. All operations were carried out at between 2 ' and 5 .

*Assay of Enzyme Activity*

MDH activity was measured at room temperature by following the formation of $NADH_2$ at 340 m$\mu$, using a Hilger "Uvispek" u.v. spectrophotometer, Model H.700. The reaction mixture contains 20 $\mu$moles of L-malate, 3 $\mu$moles of NAD, 560 $\mu$moles of phosphate buffer (pH 8·3), and enzyme solution in a final volume of 2·8 ml. The amount of enzyme used were such that the activities measured fell within the range where enzyme concentration was directly proportional to the activity. A unit of activity was defined as the amount of enzyme catalysing an extinction change at 340 m$\mu$ of 0·001/min and specific activity as the units/mg of protein.

*Estimation of Protein*

Protein was determined by the Kjeldahl method after precipitating with trichloroacetic acid or by relative measurement of light-absorption at 280 m$\mu$ when stated.

*Acknowledgements*—The author tenders his sincere thanks to Dr. B. F. Folkes for his valuable interest and encouragement throughout the course of this work and for his constructive criticism of this manuscript. He is indebted to the Inter-University Council for Higher Education Overseas for the award of a Fellowship.