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Biochem. J. (1961) **80**, 93

The Specificity of Malic Dehydrogenase from Higher Plants

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(*Received* 23 *January* 1961)

Evidence has been presented (Davies & Kun, 1957) supporting the view that malic dehydrogenase present in ox-heart mitochondria is an α-hydroxydicarboxylic acid dehydrogenase, catalysing the following reactions between substrate and diphosphopyridine nucleotide (DPN^+):

L-Malate + DPN^+
⇌ oxaloacetate + reduced DPN (DPNH) + H^+ (1)

D(−)- or *meso*-Tartrate + DPN^+
⇌ oxaloglycollate + DPNH + H^+ (2)

Oxaloglycollate + DPN^+
⇌ dioxosuccinate + DPNH + H^+ (3)

Tartronate (hydroxymalonate) + DPN^+
⇌ mesoxalate (oxomalonate) + DPNH + H^+ (4)

α-Hydroxyglutarate + DPN^+
⇌ α-oxoglutarate + DPNH + H^+ (5)

Reactions (1)–(4) have been demonstrated in higher plants and tentative evidence that separate enzymes catalyse reactions (1), (2) and (4) has been presented (Stafford, 1956, 1957). Whilst admitting that proof of specificity requires the separation of enzyme activities Stafford produced two types of evidence to support the view that malic dehydrogenase and hydroxymalonic dehydrogenase are separate enzymes: (*a*) During the purification of hydroxymalonic dehydrogenase, the relative activities with oxomalonate, dioxosuccinate and oxaloacetate were not constant; (*b*) hydroxymalonate is a competitive inhibitor of malic dehydrogenase.

It is probable that the concentration of oxomalonate and dioxosuccinate used by Stafford was well below saturation and in consequence her assays would be sensitive to errors in concentration of the unstable keto acids. The observation of competitive inhibition, contrary to Stafford's argument, supports the view that a single enzyme is involved and quantitative evidence for this view will be presented.

Delbruck, Schimassek, Bartsch & Bücher (1959) have suggested that animal cells contain two distinct malic dehydrogenases: one located in the

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mitochondria, the other being a soluble or supernatant enzyme. Malic dehydrogenase is similarly distributed in plant cells (Price & Thimann, 1954) and this paper reports a comparison of the enzymes from the two intracellular locations.

MATERIALS AND METHODS

Calcium phosphate gel. This was prepared by the method of Keilin & Hartree (1938).

Keto acids. These were obtained from L. Light and Co. Ltd. and on the basis of chromatography of their 2:4-dinitrophenylhydrazones were judged pure.

Analytical methods. Spectrophotometric measurements were made with a Unicam SP. 500 instrument, fitted with a constant-temperature compartment. Silica cells of 1 cm. light-path and 3 ml. capacity were used for the routine assay of enzyme activity, and cells of 4 cm. light-path were used for kinetic studies. DPNH was determined by measurement of light-absorption at 340 mμ. Protein was determined by the method of Warburg & Christian (1942).

Preparation of malic dehydrogenase from mitochondria and 'supernatant'. Mitochondria were isolated from the epicotyls of peas (*Pisum sativum* var. Alaska) by the method of Millerd & Bonner (1954). The mitochondrial pellet was washed three times before being suspended in potassium phosphate buffer (0·01 M, pH 7·4) and frozen by a solid carbon dioxide–acetone mixture. The extract was thawed under running tap water, and, after freezing and thawing once more, the extract was centrifuged at 30 000*g* for 15 min. The clear supernatant was collected, brought to 50% saturation with ammonium sulphate and after 10 min. the precipitated protein was collected by centrifuging, dissolved in phosphate buffer (0·2 M, pH 7·0) and dialysed for 2 hr. against the same buffer.

The supernatant obtained after removing mitochondria from the homogenate was also brought to 50% saturation and the precipitated protein dissolved and dialysed as above.

Enzyme assays. (*a*) Dehydrogenase activity was measured at 30° by following the oxidation of DPNH in silica cells of light-path 1 cm. containing potassium phosphate buffer (pH 7·0, 0·2 M), the disodium salt of ethylenediaminetetraacetic acid (3 mg.), DPNH (0·4 μmole), enzyme (0·2 ml. diluted to give an extinction change of not more than 0·07/min.) and oxaloacetate (0·5 mM), dioxosuccinate (10 mM) or oxomalonate (25 mM), in a total volume of 3 ml. Under these conditions the velocity is proportional to the enzyme concentration. A unit of activity was defined as the amount of enzyme producing an extinction change of 1·0/min. and specific activity is defined as the number of units/mg. of protein.

(*b*) Glutamic–oxaloacetic transaminase was measured at 20° by following the increase in absorption at 280 mμ in a silica cell of 1 cm. light-path containing phosphate buffer (0·2 M, pH 7·0), aspartate (0·05 M), and oxoglutarate (0·025 M), pyridoxal phosphate (0·1 mM) and enzyme in a total volume of 3 ml. Enzyme (0·1–0·5 ml.) was incubated with pyridoxal phosphate and aspartate for 10 min. before adding buffer. The reaction was started by the addition of α-oxoglutarate. Under these conditions, a unit of activity is defined as the amount of enzyme giving an extinction change of 0·01/min.

Purification of malic dehydrogenase. During the purification of glutamic–oxaloacetic transaminase from cauliflowers (R. J. Ellis & D. D. Davies, in preparation), it was observed that malic dehydrogenase was closely associated with the transaminase and in particular the two enzymes overlapped during ion-exchange chromatography on a column of *NN*-diethylaminoethylcellulose (DEAE-cellulose) (Fig. 1). After combining the fractions of the main peak of activity, a preparation from cauliflowers gave a specific activity of 100. This observation formed the basis of a method for the purification of malic dehydrogenase from pea epicotyls.

Peas were grown in darkness at 25° for 7 days, and the epicotyls were removed and blended with ice-cold potassium phosphate buffer (pH 7·4, 0·2 M) in a Waring Blendor, to give a 1:1 (w/v) homogenate. The homogenate was strained through a linen towel and after cooling to 3–5° $(NH_4)SO_4$ was added to give 50% saturation. After 10 min., the precipitated protein was collected by centrifuging at 25 000*g* for 5 min., dissolved in potassium phosphate buffer (pH 7·4, 0·2 M) and dialysed for 1 hr. against a flow of phosphate buffer (pH 7·4, 0·05 M). The solution was centrifuged clear and then serially treated with $(NH_4)_2SO_4$ to give fractions precipitated at 0–25, 25–35, 35–45 and 45–55% saturation. The precipitate from each fraction was collected by centrifuging, dissolved in phosphate buffer (pH 7·4, 0·05 M) and assayed for activity. The fraction with the main activity (35–45%) was stored overnight at −15°. After thawing, the active fraction was dialysed for 2 hr. against a flow of 15 l. of a solution of K_2HPO_4 (1 mM). Calcium phosphate gel (8·3 mg. dry wt./ml.) was added in successive small portions (0·1 vol.) and after each addition the suspension was kept for 10 min. before the gel was removed by centrifuging and the supernatant was assayed for enzyme activity. Further additions of gel were made until the specific activity of malic dehydrogenase reached a maximum. The clear solution was then poured on a column of DEAE-cellulose, previously equilibrated with a solution of K_2HPO_4 (2 mM). The column

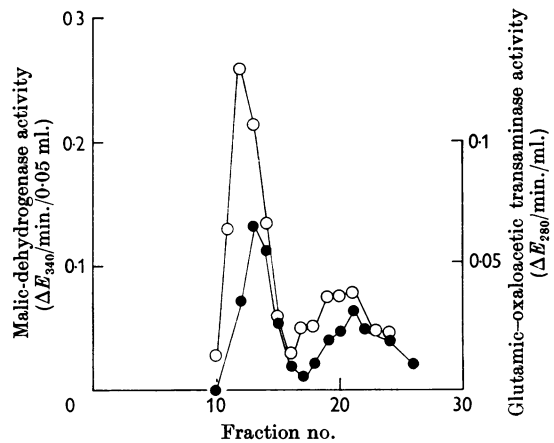


Fig. 1. Elution of cauliflower malic dehydrogenase and glutamic–oxaloacetic transaminase from a DEAE-cellulose column. ○, Malic dehydrogenase; ●, glutamic–oxaloacetic transaminase.

Table 1. *Purification of malic dehydrogenase from pea epicotyls*

Enzyme activity was measured at pH 7·0 by measuring the oxidation of DPNH by oxaloacetate at 340 mμ. A unit of activity is the amount of enzyme producing an extinction change of 1·0/min. Activity ratios for α-oxodicarboxylic acids were determined as described in the text.

Fraction	Vol. (ml.)	Total activity (units)	Total protein (mg.)	Specific activity (units/mg.)	Recovery (%)	Purification factor	Oxaloacetate / Dioxosuccinate	Oxaloacetate / Oxomalonate
Extract	1500	75 400	16 200	4·6	—	—	9·2	14·2
1st $(NH_4)_2SO_4$ precipitate	220	22 500	2 100	10·7	30	2·3	9·9	12·1
2nd $(NH_4)_2SO_4$ precipitate	50	14 200	350	40·6	19	9	10·2	12·0
Gel supernatant	72	12 300	180	67	16	15	10·2	12·7
Combined fractions from DEAE-cellulose column	60	4 100	17	240	6	52	10·0	11·9

Table 2. *Activity ratios with α-oxodicarboxylic acids*

Fraction	Specific activity	Oxaloacetate / Dioxosuccinate	Oxaloacetate / Oxomalonate
Fractions obtained during the purification of glutamate–oxaloacetate transaminase from cauliflowers			
DEAE-cellulose eluate	100	10·9	12·8
DEAE-cellulose eluate	65	10·5	12·7
Gel supernatant	10	10·0	13·2
Fractions isolated from a homogenate of pea epicotyls			
Mitochondria	58	10·5	13·7
Supernatant	4 2	9·2	12·2

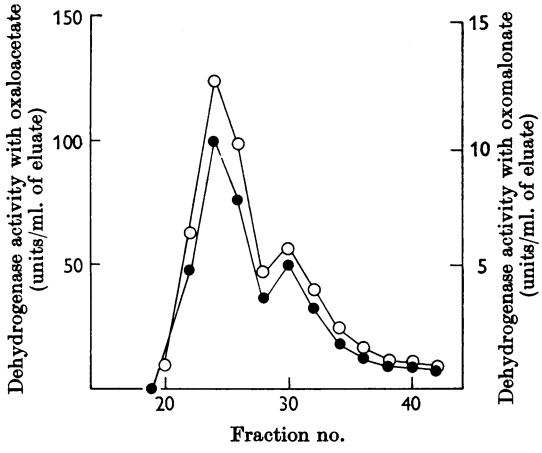


Fig. 2. Chromatography of malic dehydrogenase from pea epicotyls on DEAE-cellulose. Fractions (10 ml.) were collected and assayed for activity with oxaloacetate and oxomalonate. ○, Activity with oxaloacetate; ●, activity with oxomalonate.

was subjected to gradient elution with a mixing volume of 1 l. of K_2HPO_4 (2 mM) and a reservoir of phosphate buffer (0·2 M, pH 8·0). The column eluate was collected in 10 ml. fractions and the fractions were assayed for activity. The tubes containing the peak of enzyme activity were combined and stored at −15°; little loss of activity occurred over a period of 9 months. The results of the purification are shown in Table 1.

RESULTS

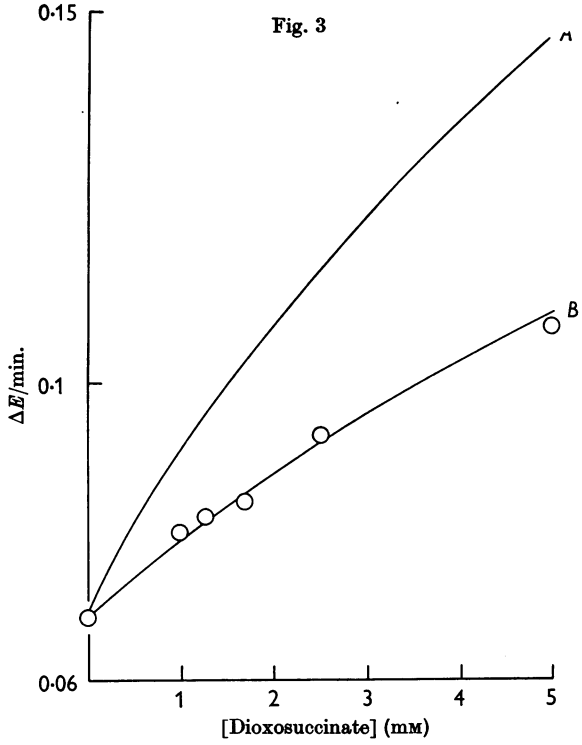
Specificity of malic dehydrogenase

Evidence that a single protein catalyses the reduction of oxaloacetate, dioxosuccinate and oxomalonate was obtained by comparing the activity ratios during purification, by comparing these ratios in preparations derived from mitochondria and supernatant, and by a kinetic study of the interaction of substrates with the enzyme.

Activity ratios. Table 1 shows that the activity ratios with different substrates were, within the limits of experimental error, constant throughout the purification. Cauliflower preparations also gave the same activity ratios (Table 2), and where two peaks of malic dehydrogenase appeared after ion-exchange chromatography (Fig. 2) the ratios were also constant.

Mixed substrates. Stafford's observation of competitive inhibition is in agreement with the hypothesis of a single enzyme. Thorn (1949) and independently Whittaker & Adams (1949) have shown that the rate of reaction with two substrates is given by the equation:

$$v_i = \frac{V_{max.}(1)\,[S_1]}{[S_1]+K_m(1)\left(1+\frac{[S_2]}{K_m(2)}\right)} + \frac{V_{max.}(2)\,[S_2]}{[S_2]+K_m(2)\left(1+\frac{[S_1]}{K_m(1)}\right)}.$$



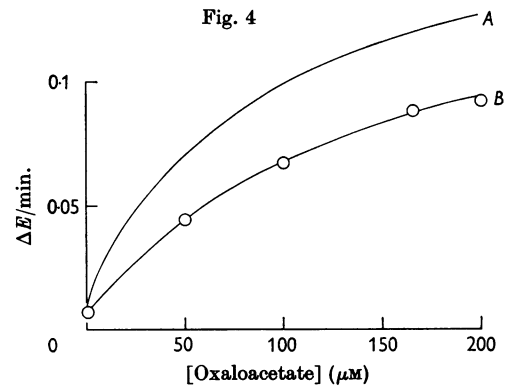


Fig. 3. Initial velocity of DPNH oxidation in the presence of cauliflower enzyme (specific activity 65), oxomalonate (10 mM), DPNH (83 μM) and dioxosuccinate (concentration as in graph). The curve for one enzyme was drawn from the equation:

$$v_t = \frac{V_{max.}(DOS) \times [DOS]}{[DOS] + K_m(DOS)\left\{1 + \frac{[OM]}{K_m(OM)}\right\}} + \frac{V_{max.}(OM) \times [OM]}{[OM] + K_m(OM)\left\{1 + \frac{[DOS]}{K_m(DOS)}\right\}}$$

$$= \frac{0{\cdot}2 \times [DOS]}{[DOS] + 8 \times 10^{-3}\left(1 + \frac{1}{2{\cdot}5}\right)} + \frac{0{\cdot}208 \times 10^{-2}}{10^{-2} + 2{\cdot}5 \times 10^{-2}\left(1 + \frac{[DOS]}{8 \times 10^{-3}}\right)}.$$

The curve for two enzymes was calculated from the equation:

$$v_t = \frac{V_{max.}(DOS) \times [DOS]}{[DOS] + K_m(DOS)} + \frac{V_{max.}(OM) \times [OM]}{[OM] + K_m(OM)}$$

$$= \frac{0{\cdot}2 \times [DOS]}{[DOS] + 8 \times 10^{-3}} + \frac{0{\cdot}208 \times 10^{-2}}{10^{-2} + 2{\cdot}5 \times 10^{-2}}$$

where DOS is dioxosuccinate and OM is oxomalonate. Experimental values are indicated by ○. *A*, Curve calculated for two enzymes; *B*, curve calculated for one enzyme.

Fig. 4. Initial velocity of DPNH oxidation in the presence of pea enzyme (specific activity 240), DPNH (83 μM), oxomalonate (0·16 mM), and oxaloacetate (concentration as in graph). The curve for one enzyme was drawn from the equation:

$$v_t = \frac{V_{max.}(OAA) \times [OAA]}{[OAA] + K_m(OAA)\left\{1 + \frac{[OM]}{K_m(OM)}\right\}} + \frac{V_{max.}(OM) \times [OM]}{[OM] + K_m(OM)\left\{1 + \frac{[OAA]}{K_m(OAA)}\right\}}$$

$$= \frac{0{\cdot}166 \times [OAA]}{[OAA] + 8 \times 10^{-5}\left(1 + \frac{1{\cdot}66}{1{\cdot}4}\right)} + \frac{0{\cdot}014 \times 1{\cdot}66 \times 10^{-2}}{1{\cdot}66 + 1{\cdot}4 \times 10^{-2}\left(1 + \frac{[OAA]}{8 \times 10^{-5}}\right)}.$$

The curve for two enzymes was drawn from the equation:

$$v_t = \frac{V_{max.}(OAA) \times [OAA]}{K_m(OAA) + [OAA]} + \frac{V_{max.}(OM) \times [OM]}{K_m(OM) + [OM]}$$

$$= \frac{0{\cdot}166 \times [OAA]}{8 \times 10^{-5} + [OAA]} + \frac{0{\cdot}014 \times 1{\cdot}66 \times 10^{-2}}{14 \times 10^{-2} + 1{\cdot}66 \times 10^{-2}}$$

where OM is oxomalonate and OAA is oxaloacetate. Experimental values are indicated by ○. *A*, Curve calculated for two enzymes; *B*, curve calculated for one enzyme.

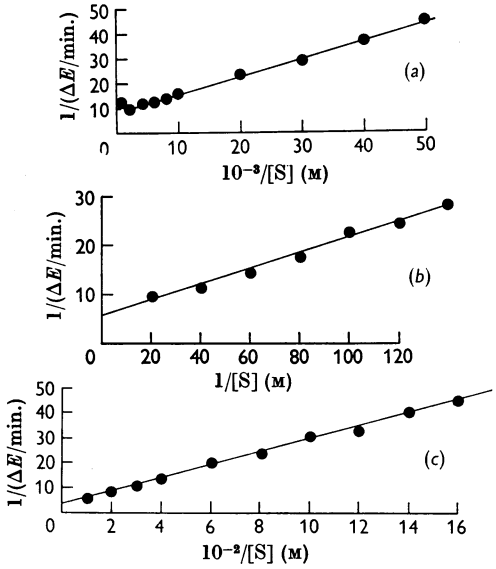


Fig. 5. Relationship between initial velocity of DPNH oxidation and concentration of keto acids. The enzyme was obtained from cauliflower and had a specific activity of 65. The activity was measured at 30° in 4 cm. cells containing DPNH (1 μmole), phosphate buffer (0·2 M, pH 7·0), keto acid as indicated and enzyme in a total volume of 12 ml. (*a*), Oxaloacetate, K_m 90 μM; (*b*), oxomalonate, K_m 25 mM; (*c*), dioxosuccinate, K_m 8 mM.

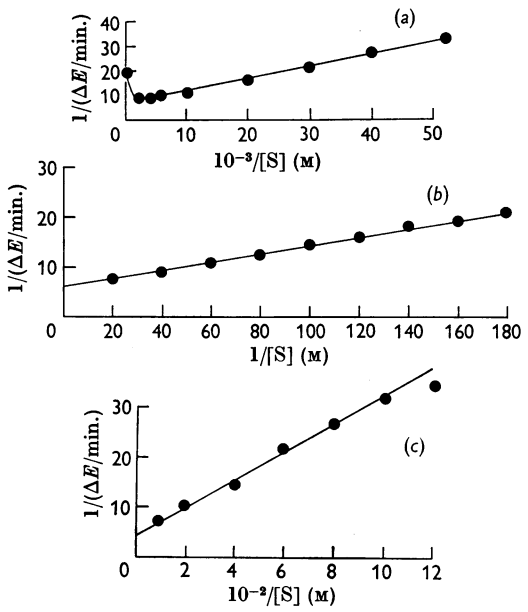


Fig. 6. Relationship between initial velocity of DPNH oxidation and concentration of keto acids. The enzyme was obtained from peas and had a specific activity of 240. The activity was measured as in Fig. 5. (*a*), Oxaloacetate, K_m 90 μM; (*b*), oxomalonate, K_m 25 mM; (*c*), dioxosuccinate, K_m 7 mM.

The rate of reaction with two substrates if separate enzymes are involved is given by:

$$v_t = \frac{V_{max.}(1)\,[S_1]}{[S_1]+K_m(1)} + \frac{V_{max.}(2)\,[S_2]}{[S_2]+K_m(2)},$$

where $V_{max.}$ represents the velocity in the presence of a saturating concentration of substrate ([S]) and K_m the Michaelis constant, i.e. the substrate concentration giving half-maximum velocity. The effect on the rate of oxidation of DPNH, when one substrate is varied and the other is kept constant, has been calculated for a single enzyme and for two enzymes. The rate determinations shown in Figs. 3 and 4 are in good agreement with the hypothesis of a single enzyme.

Kinetics. The effect of varying the substrate concentration on the rate of oxidation of DPNH was studied with the three keto acids and with enzyme preparations obtained from cauliflowers and peas. The results in Figs. 5 and 6 show that, within experimental error, the K_m values for the individual substrates are the same for enzymes prepared from cauliflowers and from peas.

The effect of varying the concentration of DPNH on the rate of reduction of oxomalonate and oxaloacetate was determined and the results are illustrated in Fig. 7; the difference between the

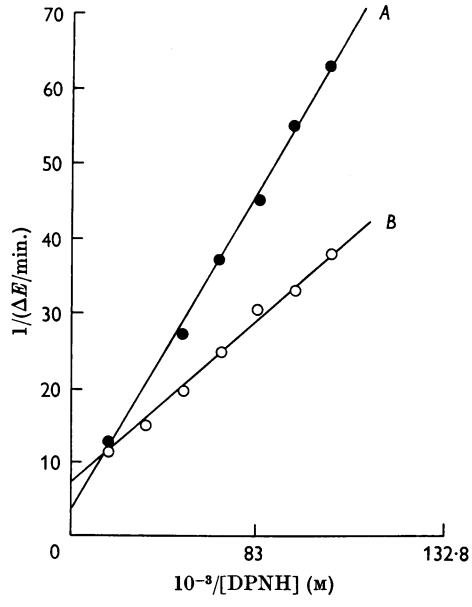


Fig. 7. Relationship between initial velocity of DPNH oxidation and concentration of DPNH, in the presence of constant concentrations of either (*A*) oxaloacetate (50 μM) or (*B*) oxomalonate (16·6 mM) and pea malic dehydrogenase (specific activity 240). The initial velocities were measured in 4 cm. cells. *A*: K_{DPNH} in the presence of oxaloacetate, 120 μM; *B*: K_{DPNH} in the presence of oxomalonate, 35 μM.

values of K_{DPNH} is greater than experimental error. Substrate inhibition was observed with oxaloacetate concentrations in excess of 0·5 mM.

Different forms of malic dehydrogenase. Preparations of malic dehydrogenase from mitochondria and supernatant were compared with respect to the effect of oxaloacetate concentration on reaction velocity. The results presented in Fig. 8 show differences between the preparations, but it is difficult to assess their significance. The possibility that malic dehydrogenase from the two cellular locations differs in substrate specificity was investigated by comparing the activity ratios. The results shown in Table 2 do not suggest any significant differences between the two preparations. The purified preparation of malic dehydrogenase was made from a homogenate in which approximately 75% of the activity was derived from the supernatant and 25% from the mitochondria.

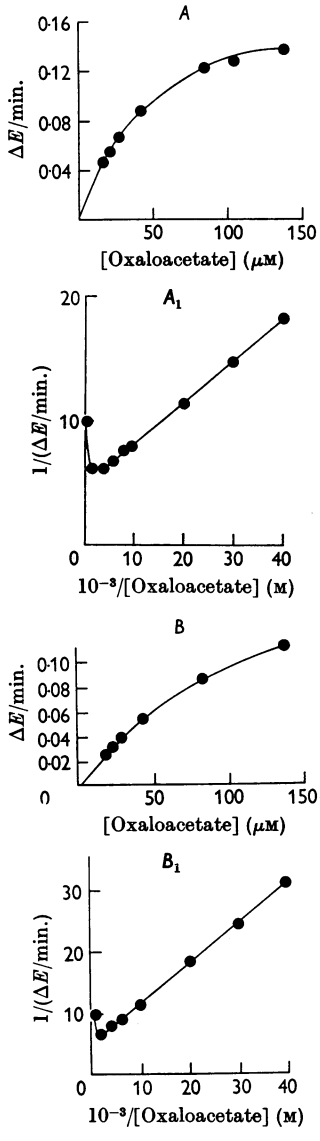


Fig. 8. Relationship between initial velocity of oxaloacetate reduction and concentration of oxaloacetate with (*A*) enzyme obtained from pea supernatant (K_m for oxaloacetate, 74 μM) and (*B*) enzyme obtained from pea mitochondria (K_m for oxaloacetate, 125 μM). The initial velocities were measured as in Fig. 5.

DISCUSSION

The experimental evidence supports the view that plant malic dehydrogenase should be considered an α-hydroxydicarboxylic acid dehydrogenase. The evidence presented by Stafford (1956) for the existence of a specific hydroxymalonic dehydrogenase may be explained as due to the failure to approach substrate saturation in the development of the enzyme assays and to a mistaken interpretation of competitive inhibition. The finding that the Michaelis constant for DPNH in the presence of oxaloacetate is not the same as in the presence of oxomalonate is not at variance with the hypothesis of a single enzyme. If K_{DPNH} represents a true dissociation constant

$$(K_{DPNH} = [E]\,[DPNH]/[E\text{–}DPNH])$$

then its value should be independent of the second substrate. However, it is probably true that in few cases is the equilibrium assumption of Michaelis & Menten (1913) completely valid and K_{DPNH} is a more complex function involving a velocity constant for the second substrate.

In recent years, attention has been drawn to the apparent heterogeneity of many enzymes and the views of a number of investigators would seem to be embodied in the following quotation: 'The concept of enzyme specificity goes far beyond substrate specificity and is to be extended to cover physiological specificity in the broadest sense' (Hess, 1958). With reference to malic dehydrogenase, electrophoresis of serum has indicated the presence of three peaks with malic-dehydrogenase activity (Vessel & Bearn, 1958). A possible physiological difference between the dehydrogenases present in the mitochondria and in the cytoplasm has been suggested by Delbruck *et al.* (1959), who confirmed the finding of Davies & Kun (1957) that the mitochondrial enzyme is inhibited by concentrations of oxaloacetate greater than 0·1 mM, and observed that the cytoplasmic dehydrogenase is not inhibited by concentrations of oxaloacetate up to 1 mM. The possibility that the kinetic differences between the enzymes is reflected in molecular structure is suggested by the

finding that, on electrophoresis, 90% of the supernatant enzyme migrates to the anode, whereas the anode component of the mitochondrial enzyme represents only 14% of the total (Wieland, Pfleiderer, Haupt & Worner, 1959). Evidence for two components with malic-dehydrogenase activity was obtained during chromatography of preparations from cauliflowers and peas (Figs. 1 and 2), but the separation did not appear to be connected with differences in specificity (Table 2).

It appears to the author that the existence of protein interactions and the likelihood of proteolytic activity modifying the structure of enzymes without destroying catalytic activity make it quite likely that electrophoresis or chromatography will indicate the presence of two peaks of activity in many enzyme preparations. The observation of multiple peaks may be unimportant unless a physiological difference between the molecular species of the enzyme is found. The existence of different enzymes catalysing the same reaction is potentially of great interest. The discovery of two examples in bacteria, the existence of two threonine deaminases, one of which is biosynthetic whereas the other is adaptive and degradative (Umbarger & Brown, 1957), and the demonstration of two distinct systems for the synthesis of acetolactate, only one of which appears to participate in valine biosynthesis (Halpern & Umbarger, 1959), suggest that the pattern of two enzymes catalysing the same reaction may be found in other biological systems.

SUMMARY

1. A method of purifying malic dehydrogenase from pea epicotyls is described. A 50-fold purification was obtained by ammonium sulphate fractionation, treatment with calcium phosphate gel and chromatography on diethylaminoethylcellulose.

2. Evidence is presented for the view that plant malic dehydrogenase, like the corresponding animal enzyme, is an α-hydroxydicarboxylic acid dehydrogenase.

3. A number of kinetic constants have been evaluated.

4. Slight differences between malic dehydrogenase prepared from mitochondria and from the supernatant remaining after removing mitochondria from a homogenate were noted. Two peaks with malic-dehydrogenase activity were observed during chromatography but all preparations appeared to have the same substrate specificity.

I wish to thank Professor T. A. Bennet-Clark, F.R.S., for advice, Mr R. J. Ellis for carrying out the assays of glutamic–oxaloacetic transaminase, Miss J. Bloom for technical assistance, and The Royal Society and the Agricultural Research Council for grants towards the cost of apparatus.

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The Determination of Magnesium in Biological Materials by Atomic Absorption Spectrophotometry

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(*Received* 28 *December* 1960)

The wide variety of methods which have been employed for the determination of magnesium in biological materials serves to illustrate the fact that no method is completely satisfactory. The majority of methods are chemical and the earlier procedures require an initial separation of magnesium, either as the insoluble ammonium phosphate salt (Briggs, 1922; Denis, 1922), or as the complex with 8-hydroxyquinoline (Greenberg & Mackey, 1932). Direct combination with the dye

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