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Properties and Regulation of Leaf Nicotinamide-Adenine Dinucleotide Phosphate-Malate Dehydrogenase and 'Malic' Enzyme in Plants with the C_4-Dicarboxylic Acid Pathway of Photosynthesis

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1. NADP–malate dehydrogenase and 'malic' enzyme in maize leaf extracts were separated from NAD–malate dehydrogenase and their properties were examined. 2. The NADP–malate dehydrogenase was nicotinamide nucleotide-specific but otherwise catalysed a reaction comparable with that with the NAD-specific enzyme. By contrast with the latter enzyme, a thiol was absolutely essential for maintaining the activity of the NADP–malate dehydrogenase, and the initial velocity in the direction of malate formation, relative to the reverse direction, was faster. 3. For the 'malic' enzyme reaction the K_m for malate was dependent on pH and the pH optimum varied with the malate concentration. At their respective optimum concentrations the maximum velocity for this enzyme was higher with Mg^{2+} than with Mn^{2+}. 4. The NADP–malate dehydrogenase in green leaves was rapidly inactivated in the dark and was reactivated when plants were illuminated. Reactivation of the enzyme extracted from darkened leaves was achieved simply by adding a thiol compound. 5. The activity of both enzymes was low in etiolated leaves of maize plants grown in the dark but increased 10–20-fold, together with chlorophyll, when leaves were illuminated. 6. The activity of these enzymes in different species with the C_4-dicarboxylic acid pathway was compared and their possible role in photosynthesis was considered.

In plants with the C_4-dicarboxylic acid pathway of photosynthesis (Hatch & Slack, 1970*a*,*b*) fixed CO_2 appears initially in the dicarboxylic acids malate, aspartate and oxaloacetate (Hatch & Slack, 1966; Hatch, Slack & Johnson, 1967). Oxaloacetate, formed by phosphopyruvate carboxylase, is the first product of CO_2 fixation (Slack & Hatch, 1967). Although the content of 'malic' enzyme (EC 1.1.1.40) in leaves of at least some plants with the C_4-dicarboxylic acid pathway is about 45-fold higher than that in Calvin-cycle plants it probably does not contribute significantly to the fixation of CO_2 (Slack & Hatch, 1967; Slack, Hatch & Goodchild, 1969). Rather, malate is formed from oxaloacetate by a unique malate dehydrogenase specific for NADP (Hatch & Slack, 1969*a*). Both NADP–malate dehydrogenase and 'malic' enzyme are located in chloroplasts, the former enzyme being exclusively associated with the mesophyll chloroplasts and the latter with the chloroplasts of the bundle sheath cells (Slack *et al.* 1969). The possibility has been considered that 'malic' enzyme may operate to decarboxylate malate transported from the mesophyll chloroplasts and that the CO_2 released is refixed by ribulose diphosphate carboxylase (EC 41.13.9) (Slack *et al.* 1969; Hatch & Slack, 1970*a*). The purpose of the present study was to examine the properties and regulation of NADP–malate dehydrogenase and 'malic' enzyme.

MATERIALS

Leaves were obtained from maize (*Zea mays*, var. D5606A) or other plants grown in a glasshouse at 28°C. Tricine,† HEPES and MES buffers, dithiothreitol and

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† Abbreviations: tricine, *N*-tris(hydroxymethyl)-methylglycine; HEPES, 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid; MES, 2-(*N*-morpholine)-ethanesulphonic acid.

aspartate aminotransferase were obtained from Calbiochem, Los Angeles, Calif., U.S.A. Other biochemicals and enzymes were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

METHODS

Extraction and preparation of enzymes

Crude extracts. The content of enzymes in leaves was determined by grinding weighed samples of leaf in a chilled mortar for 90s with sand and 4vol. (w/v) of 0.1M-tris–HCl buffer, pH8.3, containing 5mM-dithiothreitol and 2mM-EDTA. This and subsequent steps were conducted at 3°C. For studies on the activation and inactivation of NADP–malate dehydrogenase in leaves, where rapid assay was essential, clear extracts were obtained by filtering through Whatman no. 1 paper. For other studies, the extracts obtained by straining the homogenates through Mira-cloth were treated on a small column of Sephadex G-25 to remove low-molecular-weight materials. The Sephadex was pre-equilibrated with 25mM-tris–HCl buffer, pH8.3, and 5mM-dithiothreitol and 1mM-EDTA were also included as desired. Clear extracts were obtained by centrifuging at 10000*g* for 5min.

Partial purification of enzymes. Maize leaf (150g) was blended for 3min at maximum speed in a Servall Omnimix with 600ml of 0.1M-tris–HCl buffer, pH8.3, containing 50mM-2-mercaptoethanol, 5mM-$MgCl_2$ and 5mM-EDTA. The temperature was maintained at 0°C and the gas atmosphere was N_2. Subsequent steps were performed at 3°C. The extract obtained by filtering through muslin was centrifuged at 10000*g* for 10min and the supernatant was treated with saturated $(NH_4)_2SO_4$ (pH7.0, 4°C) to obtain the protein fraction precipitating between 50% and 55% saturation. This protein was dissolved in 50mM-tris–HCl buffer, pH8.0, containing 5mM-dithiothreitol and 2mM-EDTA, and incubated for 1h at 30°C to restore the maximum activity of NADP–malate dehydrogenase. A 4.5ml sample of this solution was applied to a 170ml column (2.5cm diam.) of superfine Sephadex G-200 that had been previously equilibrated with a solution containing 25mM-tris–HCl buffer, pH8.0, 2.5mM-dithiothreitol and 2.5mM-EDTA. Fractions (2.8ml) were collected at a flow rate of 0.1ml/min. The temperature for this treatment was 22°C. The protein in active fractions was stored as a precipitate at 3°C in a 70%-saturated solution of $(NH_4)_2SO_4$. As required, samples of this suspension were centrifuged and the precipitated protein was dissolved in 25mM-tris–HCl buffer, pH8.0, containing 5mM-dithiothreitol, 5mM-$MgCl_2$ and 2.5mM-EDTA. For studies with NADP–malate dehydrogenase this solution was incubated at 30°C to restore full activity.

Assay of enzymes

Malate dehydrogenases. For the direction towards malate formation the decrease in E_{340} was measured in reaction mixtures containing enzyme, either 25mM-tricine–NaOH or tris–HCl buffer, pH8.0, 1mM-EDTA, 0.5mM-oxaloacetate and either 0.2mM-NADH or NADPH. For the direction towards oxaloacetate formation the increase in E_{340} was measured in reactions containing enzyme, 25mM-tricine–NaOH buffer, pH8.9, 1mM-EDTA, 1mM-$NADP^+$, 50mM-malate, 20mM-glutamate and 0.6 unit/ml of aspartate aminotransferase. The temperature for assay was 22°C.

'Malic' enzyme. The increase in E_{340} was measured at 22°C in reactions containing enzyme, either 25mM-tricine–NaOH buffer, pH8.5, or 25mM-tris–HCl buffer, pH8.0, 0.5mM-EDTA, 2.5mM-malate, 0.25mM-$NADP^+$ and either 5mM-$MgCl_2$ or $MnCl_2$ at the concentration indicated in the text. Reactions were started by adding $MgCl_2$ or $MnCl_2$ so that any blank rate due to NADP–malate dehydrogenase could be determined. This rate was negligible.

Oxaloacetate decarboxylase (*EC* 4.1.1.3). The assay procedure employed was essentially that described by Kosicki (1968). Reactions contained enzyme, 25mM-sodium acetate buffer, pH5.3, 5mM-$MnSO_4$ and 0.75mM-oxaloacetate.

Reaction products and stoicheiometry

NADP–malate dehydrogenase. Partially purified enzyme was incubated in reaction mixtures containing 0.2mM-[4-^{14}C]oxaloacetate and an excess of NADPH until there was no further change of E_{340}. After addition of $HClO_4$ to give a final concentration of 2% (w/v) the radioactive products were chromatographed on paper and identified as described by Hatch & Slack (1966).

For determining the stoicheiometry of the reaction the enzyme was incubated with 0.25mM-tris–HCl buffer, pH8.0, 1mM-EDTA, 0.1mM-NADPH and 0.77μM-oxaloacetate until there was no further change in E_{340}. The amount of malate formed was determined by adding partially purified malic enzyme from maize together with 5mM-$MgCl_2$ and 0.25mM-$NADP^+$. The increase in E_{340} provided a measure of the malate present, this being verified by adding samples of standard malate.

'Malic' enzyme. Partially purified 'malic' enzyme was incubated with 25mM-tris–HCl buffer, pH8.0, 0.5mM-EDTA, 5mM-$MgCl_2$, 0.25mM-$NADP^+$ and 0.1mM-malate. When there was no further change of E_{340}, $HClO_4$ was added to give a final concentration of 1% (w/v). The mixture was cooled to 0°C, neutralized with KOH and the precipitate of $KClO_4$ was centrifuged. The other product, pyruvate, was identified by adding lactate dehydrogenase and NADH to a sample of the supernatant.

Other procedures

The artificial light sources and the procedures for measuring light-intensity were as previously described (Hatch, Slack & Bull, 1969). Protein was determined by the method of Warburg & Christian (1941) and chlorophyll by the method of Arnon (1949).

RESULTS

Extraction and partial purification of enzymes

When thiol compounds, either dithiothreitol or 2-mercaptoethanol, were omitted from the extraction medium less than 5% of the NADP–malate dehydrogenase activity was recovered. Mg^{2+} had

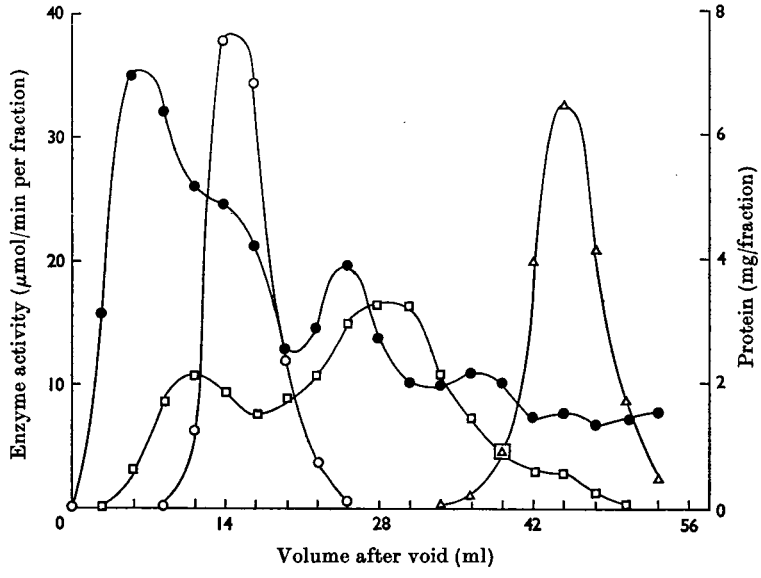

Fig. 1. Chromatography of malate dehydrogenases and 'malic' enzyme on Sephadex G-200. Details of the procedure are described in the Methods section. The elution profiles are shown for protein (●), 'malic' enzyme (○), NADP–malate dehydrogenase (□) and NAD–malate dehydrogenase (△).

a marginally beneficial effect and was generally also included in the extraction medium. Provided the oxidation of thiol compounds by oxygen was prevented the extracted enzyme remained active for several hours at 0°C. 'Malic' enzyme activity was not increased by including thiol compounds in the extraction medium. Both enzymes retained activity for several months when stored as precipitates in a 66%-saturated solution of ammonium sulphate at 3°C.

NADP–malate dehydrogenase and 'malic' enzyme were partially purified and separated from NAD–malate dehydrogenase by the procedure described in the Methods section. Although about half of the activity of the former enzymes precipitated in the 55–65%-saturated ammonium sulphate fraction the 50–55% fraction was used because it excluded most of the NAD–malate dehydrogenase. The result of the chromatography of the latter fraction on Sephadex G-200 is shown in Fig. 1. 'Malic' enzyme emerged as a single peak just after the peak of protein not retarded by the gel. There were two major peaks of NADP–malate dehydrogenase activity and these were distinct from the peaks of both 'malic' enzyme and NAD–malate dehydrogenase. Further information about the relationship between these two components is presented below. For most of the studies described below the lower-molecular-weight species of NADP–malate dehydrogenase was used.

Properties of NADP–malate dehydrogenase

Products and stoicheiometry. When [4-^{14}C]-oxaloacetate was incubated with the enzyme and an excess of NADPH the radioactive product was identified as malate by the procedures described in the Methods section. To confirm this result and establish the stoicheiometry of the reaction, 77nmol of oxaloacetate was incubated with the enzyme and an excess of NADPH as described in the Methods section. When the reaction was completed 74nmol of NADPH had been oxidized and 80nmol of malate was formed. A similar result was obtained with the higher-molecular-weight species of the enzyme. Activity with NADH was undetectable. Accordingly, the systematic name for this enzyme would be L-malate–NADP oxidoreductase.

Effect of pH. NADP–malate dehydrogenase activity was close to optimum over relatively wide pH ranges but the optimum varied with the oxaloacetate concentration (Fig. 2). With oxaloacetate concentrations of 0.05mM and 2mM the optima were pH 8.1 and pH 8.9 respectively.

Michaelis constants and forward and reverse velocities. Lineweaver–Burk plots for oxaloacetate and for NADPH were linear and gave K_m values of 25μM and 60μM respectively. These values were determined at pH 8.0 and with 0.2mM of the other substrate in each case. For the reaction in the

reverse direction the plots of activity against both malate and NADP concentration were sigmoidal in shape rather than hyperbolic (Fig. 3). The concentrations of malate and NADP giving half-maximal activity, determined from these plots, were about 2mM and 0.25mM respectively.

We previously reported that the initial rate of the reaction catalysed by NADP–malate dehydrogenase in the direction of oxaloacetate formation was about 30 times that in the reverse direction at pH 8.9 (Hatch & Slack, 1969*a*). However, the above results show that relatively high concentrations of malate and NADP are required for maximum activity in the reverse direction. With near-saturating concentrations of these reactants the rate in the forward direction was only about nine times that for the reverse direction.

Different-molecular-weight forms of the enzyme. From the data of Andrews (1965) we calculated that the faster-moving peak of NADP–malate dehydrogenase on Sephadex G-200 (Fig. 1) had approximately twice the molecular weight of the slower-moving peak. A third and minor component of activity, with a mobility similar to that of NAD–malate dehydrogenase, was observed on both occasions that this procedure was performed. A sample of the most active fractions of the high-molecular-weight peak was rechromatographed on Sephadex G-200 after the enzyme had been precipitated and stored as a suspension in ammonium sulphate solution at 3°C. All the activity emerged in the position of the two slower-moving, lower-molecular-weight species, activity being about equally distributed between these species. It was not determined whether the transformation of the higher-molecular-weight species to the lower-molecular-weight forms only occurred if the enzyme was precipitated and stored in ammonium sulphate solution.

Effect of metabolites on activity. The effect of various photosynthetic intermediates as activators or inhibitors of NADP–malate dehydrogenase was tested. At least at a concentration of 1mM the mono- and di-phosphates of ribulose and fructose, phosphoenolpyruvate, dihydroxyacetone phosphate, 3-phosphoglycerate and orthophosphate were without effect. Similarly, they had no influence on 'malic' enzyme activity.

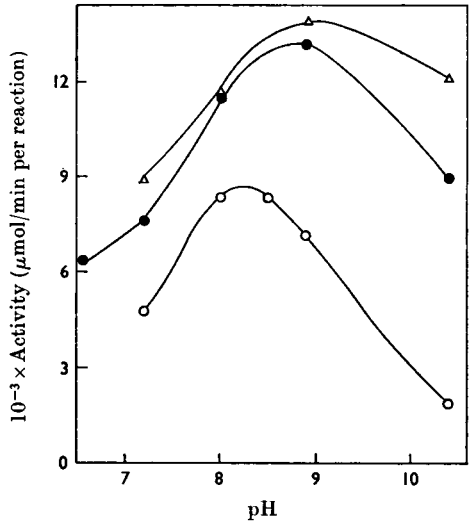

Fig. 2. Effect of pH on the activity of NADP–malate dehydrogenase at different concentrations of oxaloacetate. The assay procedure was as described in the Methods section, except that the buffers used (at a concentration of 25mM) were MES–NaOH for pH 6.5, tris–HCl for pH 7.2, 8.0 and 8.9 and glycine–NaOH for pH 10.4. The concentrations of oxaloacetate used were 0.05mM (○), 0.5mM (●) and 2mM (△).

Properties of 'malic' enzyme

Requirements and stoicheiometry. The enzyme catalysed the reduction of $NADP^+$ in the presence of malate and a divalent metal ion. At their respec-

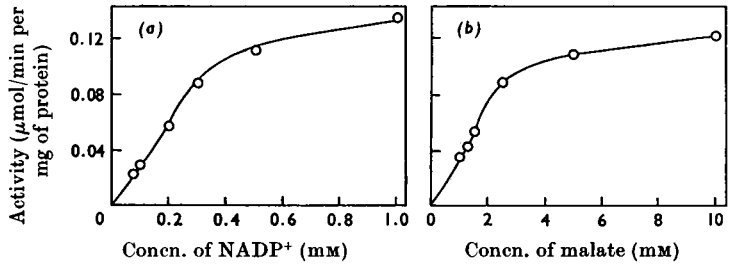

Fig. 3. Effect of the concentration of $NADP^+$ and malate on NADP–malate dehydrogenase activity in the direction of oxaloacetate synthesis. Reaction mixtures were as described in the Methods section except that $NADP^+$ (*a*) and malate (*b*) were varied as shown.

tive optimum concentrations the enzyme was about 30% more active with Mg^{2+} than with Mn^{2+}. There was no activity with NAD^+. When 0.1 μmol of malate was provided with an excess of $NADP^+$ as described in the Methods section the reduction of 0.093 μmol of $NADP^+$ was recorded. The ratio of activities in the forward and reverse directions at pH 8.0, with 40mM-sodium hydrogen carbonate, 20mM-pyruvate, 0.15mM-NADPH and 5mM-magnesium chloride in the reverse direction assay system, was about 4.

Effect of malate on the pH *optimum.* The response of 'malic' enzyme activity to changes in pH varied with the malate concentration (Fig. 4). With malate at concentrations of 0.1mM, 1mM and 10mM the respective optima were about pH 7.4, 8.0 and 8.5. Further evidence is provided below that the interaction between malate concentration and pH is complex.

Michaelis constants. The apparent Michaelis constant for malate varied with the pH (Fig. 5), the values determined from Lineweaver–Burk plots being 0.4mM at pH 8.5 and 0.15mM at pH 8.0. At pH 7.5 malate inhibited activity at concentrations of more than 1mM. A similar effect was observed with bacterial 'malic' enzyme (Parvin, Pande & Venkitasubramanian, 1964).

At pH 8.0 and with 2.5mM-malate the K_m for $NADP^+$ was 25 μM. The values for Mg^{2+} and Mn^{2+} were 55 μM and 4 μM respectively with 10mM-malate and 0.25mM-$NADP^+$ at pH 8.5, and with optimum concentrations of Mg^{2+} and Mn^{2+} the respective maximum velocities were 3.7 and 3.0 μmol/min per mg of protein. A similar K_m was obtained for Mn^{2+} with 1mM-malate at pH 7.5 but the K_m for Mg^{2+} under these conditions was 570 μM.

Oxaloacetate decarboxylase activity. Like 'malic' enzyme from other sources (Kun, 1963) there was oxaloacetate decarboxylase activity associated with the maize enzyme. The decarboxylating activity of fractions from Sephadex G-200 with the highest 'malic' enzyme activity (Fig. 1) was optimum at pH 5.7. Only about 20% of this activity was observed at pH 4.5 and pH 6.7. At their respective optimum conditions the rate of malate decarboxylation by 'malic' enzyme was about twice that for decarboxylation of oxaloacetate.

Enzyme activity in different species

NADP–malate dehydrogenase activities of about 10–12 μmol/min per mg of chlorophyll have now been recorded for sugar-cane and maize leaves, about twice those previously reported (Hatch & Slack, 1969*a*). Activities in other species with the C_4-dicarboxylic acid pathway, *Amaranthus edulis*, *Gomphrena celosoides*, *Chloris gayana*, *Atriplex nummularia* and *Eragrostis brownii* varied between 1 and 4 μmol/min per mg of chlorophyll. The activities for 'malic' enzyme in leaves of sugar-cane, maize and *Gomphrena celosoides* were 12, 10 and 5 μmol/min per mg of chlorophyll but for the other species mentioned above the values varied between 0.2 and 0.8.

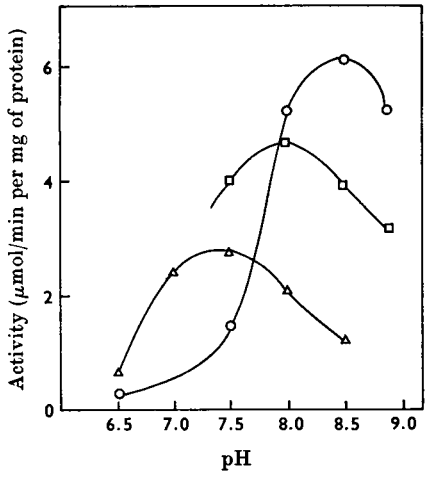

Fig. 4. Effect of pH on the activity of 'malic' enzyme at different concentrations of malate. Reaction mixtures were as described in the Methods section except that the following buffers were used (at 25mM): MES–NaOH for pH 6.5, HEPES–NaOH for pH 7.0 and 7.5 and tricine–NaOH for pH 8.0, 8.5 and 8.9. The malate concentrations used were 10mM (○), 1mM (□) and 0.1mM (△).

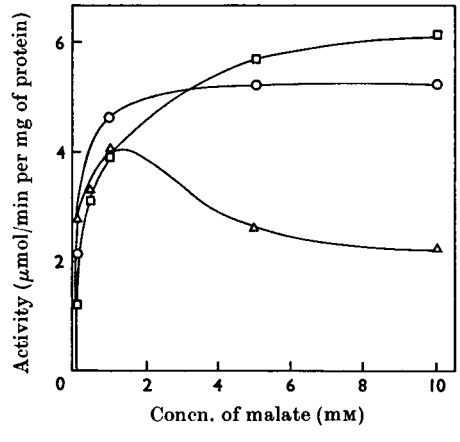

Fig. 5. Effect of malate concentration on 'malic' enzyme activity at different values of pH. Assay conditions were as described in the Methods section with tricine–NaOH buffer at pH 7.5 (△), pH 8.0 (○) and pH 8.5 (□).

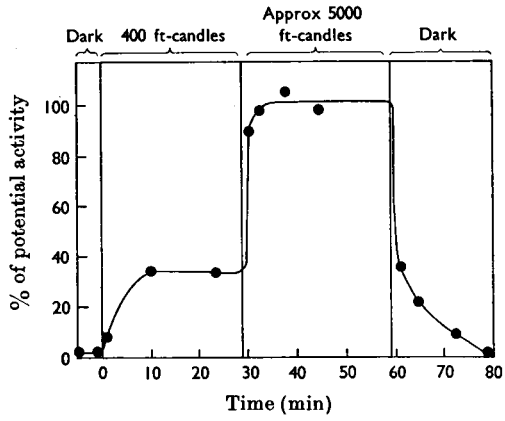

Fig. 6. Effect of light-intensity on the activity of NADP–malate dehydrogenase in maize leaves. Plants were grown at 28°C with natural illumination and before the experiment were illuminated until 10 a.m. and then transferred into the dark for 30min. The activity of the enzyme before transfer was equivalent to 100% on the ordinate. After 30min in darkness, zero on the time-scale, plants were transferred successively to the light conditions indicated and samples of leaves were extracted and assayed as described in the Methods section.

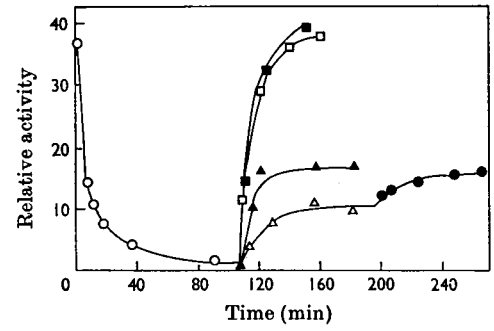

Fig. 7. Inactivation and activation of NADP–malate dehydrogenase in extracts. The enzyme was extracted from illuminated leaves as described in the Methods section and then freed of dithiothreitol and EDTA by treatment on a Sephadex G-25 column previously equilibrated with 25mM-tris–HCl buffer, pH 8.3. This extract was incubated at 0°C for 110min (○). At this time samples were incubated with either 5mM-dithiothreitol at 30°C (□) or 0°C (△) or with 5mM-dithiothreitol plus 5mM-$MgCl_2$ at 30°C (■) or 0°C (▲). At 200min the treatment at 0°C with dithiothreitol was supplemented with 5mM-$MgCl_2$ (●).

Factors affecting enzyme activity in vivo *and* in vitro

Light-dependent regulation of NADP–malate dehydrogenase. After maize plants had been kept in darkness for 30min the activity of NADP–malate dehydrogenase in leaf extracts was only about 2% of that for plants illuminated in sunlight (Fig. 6). Upon transfer from darkness to illumination at 400 ft-candles, about 3% of full sunlight, activity increased about 17-fold in 10min. A further threefold increase occurred when plants were returned to daylight and activity declined again when plants were returned to darkness. The daylight intensity in the experiment described in Fig. 6 varied between 2000 and 8000 ft-candles due to the prevailing cloudy conditions. Although extracts were prepared at 0°C and rapidly assayed thereafter, the activity recorded for darkened leaves was probably due to partial activation of the enzyme during extraction. When extracts from leaves of darkened plants were incubated with 5mM-dithiothreitol for 30min at 30°C full activity was restored. 'Malic' enzyme activity was not affected by changes in the light-intensity.

Studies in extracts. When leaf extracts from illuminated plants were depleted of dithiothreitol the activity of NADP–malate dehydrogenase declined to zero after about 25min at 30°C. Adding dithiothreitol at this time restored activity after a further 40min. When the addition of dithiothreitol was further delayed only part of the activity was recovered. However, particularly under the latter conditions, the rate of activation was increased as much as 50% if 5mM-$MgCl_2$ was also provided. Under similar conditions the partially-purified enzyme was also inactivated but no reactivation occurred when dithiothreitol and $MgCl_2$ were added.

Inactivation was slower at 0°C (Fig. 7). However, full activity was recovered by adding back dithiothreitol after 110min provided the temperature was increased to 30°C for the reactivation treatment. Only about 25% of the original activity was recovered at 0°C and about 43% if $MgCl_2$ was also provided. Addition of $MgCl_2$ to the system originally containing only dithiothreitol at 0°C resulted in a further increase in activity. If the temperature was increased to 30°C after this increase no additional activity was recovered.

Enzyme formation in greening leaves. The activity of several enzymes of the C_4-dicarboxylic acid pathway increase manyfold when etiolated leaves are illuminated (Hatch *et al.* 1969). Chloramphenicol, an inhibitor of protein synthesis by chloroplasts, prevents these increases (Graham, Hatch, Slack & Smillie, 1970). When etiolated maize plants were illuminated at 1500 ft-candles the activity of NADP–malate dehydrogenase and 'malic' enzyme, expressed per unit fresh weight, increased 10–20-fold as greening occurred. At the same time there was no significant change in NAD–malate dehydrogenase activity. To confirm that

the increase in NADP–malate dehydrogenase activity was not due simply to activation of existing enzyme, extracts were preincubated with dithiothreitol before assay of the enzyme.

DISCUSSION

There is ample evidence that NADP–malate dehydrogenase and 'malic' enzyme are implicated in photosynthesis in plants with the C_4-dicarboxylic acid pathway. Results from earlier studies (Slack & Hatch, 1967; Hatch & Slack, 1969*a*), together with those provided during the present investigation, show that the activity of these enzymes in most plants with this pathway is much higher than in Calvin-cycle plants. Further, these enzymes are associated with chloroplasts (Slack *et al.* 1969) and, as shown in the present studies, are synthesized as chloroplasts are formed in greening leaves.

NADP–malate dehydrogenase is located in the mesophyll chloroplasts (Slack *et al.* 1969), together with the other enzymes of the first cycle of the C_4-dicarboxylic acid pathway (Hatch & Slack, 1970*a*), and may reasonably be assumed to account for the conversion of oxaloacetate into malate. However, the fate of the malate so formed is uncertain. Evidence summarized in a recent review (Hatch & Slack, 1970*a*) favoured the proposal that C-4 of a dicarboxylic acid, possibly oxaloacetate, was transferred by a transcarboxylation reaction to an acceptor to provide C-1 of 3-phosphoglycerate. If this process was operative malate would appear to serve only as a reservoir pool in reversible equilibrium with oxaloacetate. An alternative proposal was that malate may move from the mesophyll chloroplasts to the bundle-sheath chloroplasts and there undergo decarboxylation by 'malic' enzyme (Slack *et al.* 1969; Hatch & Slack, 1970*a*). The CO_2 so released could then be refixed by ribulose diphosphate carboxylase. The apparently low activities of the latter enzyme in C_4-dicarboxylic acid pathway plants was one of the reasons why this route was considered to be of only minor importance. However, Bjorkman & Gauhl (1969) have now shown that the activity of ribulose diphosphate carboxylase in plants with this pathway are comparable with those for Calvin-cycle plants. Andrews & Hatch (1970) have confirmed this observation and attributed the discrepancy partly to incomplete extraction of the enzyme and partly to the use of non-optimum assay conditions. These observations eliminate one of the major objections to the operation of the route for C-4 carboxyl transfer involving 'malic' enzyme. The significance of the fact that some plants with the C_4-dicarboxylic acid pathway have relatively low 'malic' enzyme activity remains to be determined.

Some of the properties of NADP–malate dehydrogenase and 'malic' enzyme have possible relevance to their function or regulation *in vivo*. The sigmoid shape of the plots of NADP–malate dehydrogenase activity with varying malate and $NADP^+$ concentrations suggest that these substrates have an activating effect on the enzyme in the direction of malate oxidation. The fact that the pH optima for both enzymes varied with substrate concentration may also have regulatory significance. In addition, the K_m for malate in the reaction catalysed by 'malic' enzyme varied with pH. It was reported earlier that the rate of the NADP–malate dehydrogenase reaction in the direction of malate formation was 30 times as fast as the reverse rate (Hatch & Slack, 1969*a*). However, the present studies demonstrated that relatively high concentrations of malate and $NADP^+$, about 50mM and 1mM respectively, are required for optimum activity in the latter direction. Even with such concentrations in the reverse-direction assay the forward direction was still nine times as fast. This contrasts with NAD–malate dehydrogenase, for which the rates in the two directions are about equal at higher pH values (Raval & Wolfe, 1962). If the arguments of Cleland (1967) are applied it might be concluded that the NADP–malate dehydrogenase is designed to operate primarily in the direction of malate formation. This would favour the view that malate is the C_4-dicarboxylic acid that provides the C-4 carboxyl group for 3-phosphoglycerate formation.

NADP–malate dehydrogenase activity is apparently regulated *in vivo* by the prevailing light-intensity in a manner similar to that described for pyruvate,P_i dikinase (Slack, 1968; Hatch & Slack, 1969*b*). As for the latter enzyme, the regulation of NADP–malate dehydrogenase would probably be of importance not only during transitions between dark and light but also as the light-intensity varies during the day. The mechanism of the effect was apparently also similar for the two enzymes, involving the reversible oxidation of vicinal thiol groups, but the requirements for activation and inactivation of NADP–malate dehydrogenase *in vitro* were relatively simple compared with pyruvate, P_i dikinase (Hatch & Slack, 1969*b*). However, isolated NADP–malate dehydrogenase can exist in three interconvertible molecular-weight forms and this may be related to the differing activation of the enzyme observed when temperature and Mg^{2+} concentration were varied. The relation between these forms and the regulation of the enzyme *in vivo* was not established.

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