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MALATE DEHYDROGENASE IN LEAF PEROXISOMES

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SUMMARY

The technique of isopycnic centrifugation of leaf homogenates has allowed a separation of chloroplasts, mitochondria, and peroxisomes according to their respective densities. Chlorophyll, cytochrome *c* oxidase, and glycolate oxidase, respectively, were used as markers for these organelles. Malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37) showed peaks of activity in the mitochondrial and peroxisomal fractions. The peroxisomal enzyme as assayed by following reduced pyridine nucleotide oxidation had a broad pH optimum from 6.4 to 7.4 and was specific for NADH. The peroxisomal and mitochondrial forms of malate dehydrogenase were differentiated by their kinetic and electrophoretic behavior. The mitochondrial form had a K_m (oxalacetate) of $5.7 \cdot 10^{-6}$ M and was inhibited by oxalacetate concentrations above $7 \cdot 10^{-5}$ M. The peroxisomal form had a K_m (oxalacetate) of $1.4 \cdot 10^{-5}$ M and was inhibited by oxalacetate concentrations in excess of $2 \cdot 10^{-4}$ M. The supernatant malate dehydrogenase showed kinetic characteristics intermediate to those of the peroxisomal and mitochondrial forms. Starch-gel electrophoresis of the supernatant fraction showed the presence of both the peroxisomal and mitochondrial forms together with another isozyme.

INTRODUCTION

Although the presence of multiple forms of malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37) in leaf tissue is a well-established fact, there are conflicting reports concerning the location(s) of the nonmitochondrial form(s). Early reports[1,2] excluded the possibility of malate dehydrogenase activity in the chloroplast, whereas later reports[3–9] have localized activity in the chloroplasts or plastid fraction. In addition several workers have characterized 'soluble' malate dehydrogenases from leaf tissue and have shown these forms to be kinetically and electrophoretically distinct from the mitochondrial form[10–12].

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

Our laboratory has recently reported the isolation and partial characterization of peroxisomes from various leaf tissues[13–15]. The peroxisomes were shown to contain the enzymes glycolate oxidase, NAD glyoxylate reductase, and catalase. This report describes the presence and some of the properties of the particulate malate dehydrogenase in leaf peroxisomes.

MATERIALS AND METHODS

Spinach (*Spinacia oleracea* L.) was purchased locally. The leaf tissue was washed, deribbed, and cut into small strips. Subsequent operations were carried out at 0–4° either in a cold room or in ice buckets. The tissue (100 g) was homogenized in a Waring blender for 10 sec with 1 vol. by weight of grinding medium (0.5 M sucrose, 0.02 M potassium glycyl-glycine, pH 7.5). The resulting slurry was squeezed through twelve layers of cheesecloth, and the pH (about 7) was readjusted to 7.5 with KOH. The sap was then centrifuged at 100 × *g* for 20 min, and the resulting pellet resuspended in the grinding medium and designated the 'Chloroplast' fraction. The sap was further centrifuged at 6000 × *g* for 20 min, and this pellet, after resuspension in the grinding medium, was designated the 'Peroxisome and broken chloroplast' fraction, for it contained besides the peroxisomes large amounts of chloroplast fragments and some mitochondria. A 'Mitochondrial' fraction was prepared by centrifugation at 37 000 × *g* for 20 min and resuspension of the pellet. The supernatant fluid after the last centrifugation was designated the 'Supernatant' fraction. Preparations were made with other plants using substantially the same procedure, although in some cases the volume of the grinding medium was increased to facilitate homogenization.

A discontinuous sucrose density gradient was prepared in the cold by pipetting successively 4 ml of 2.5 M, 8 ml of 2.3 M, 10 ml of 1.8 M, 15 ml of 1.5 M, and 13.5 ml of 1.3 M sucrose in a cellulose nitrate tube designed for the Spinco SW 25.2 swinging-bucket rotor (all sucrose solutions were 0.02 M in potassium glycyl-glycine, pH 7.5). A portion of the 'Peroxisomal and broken chloroplast' fraction (4 ml) was then layered on top of the gradient, and the sample was centrifuged in a Spinco Model L centrifuge at 25 000 rev./min for 3 h at 4°. Fractions were collected from the bottom of the tube after puncturing and are numbered in the order of collection.

Malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37) was assayed spectrophotometrically by following the oxidation of NADH at 340 mμ using a Gilford automatic recording spectrophotometer. The assay mixture contained 0.67 ml of 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4), 0.03 ml of 0.5% Triton X-100, 0.04 ml of 0.01 M oxalacetic acid, neutralized to pH 7.4 with KOH, 0.02 ml of $2.81 \cdot 10^{-3}$ M NADH, and enzyme *plus* water to give a total volume of 1 ml in the cuvette. The reaction was initiated by the addition of oxalacetate. A unit of activity has been defined as the oxidation of 1 μmole NADH per min at 25°.

Glycolate oxidase (EC 1.1.3.1) was used as a marker for peroxisomes and was assayed by following the anaerobic reduction of 2,6-dichlorophenolindophenol as described previously[14]. Cytochrome *c* oxidase (EC 1.9.3.1) was used as a marker for mitochondria and was assayed by following the oxidation of reduced cytochrome *c*

(ref. 14). Chlorophyll was estimated by the method of ARNON[16] and protein by the method of LOWRY[17].

Starch-gel electrophoresis was carried out following the method of FINE AND COSTELLO[18] using Triton X-100 extracts of the fractions. Clear separation of the isozymes was noted after electrophoresis at 27 mA for 18 h at 4°. Enzymatic activity on the gel was detected by dark incubation in a buffer containing malate, NAD^+, phenazine methosulfate, and Nitro Blue Tetrazolium for 30 min at 37° as described by FINE AND COSTELLO[18].

Crystalline glyoxylate reductase (glycolate:NAD oxidoreductase, EC 1.1.1.26) was purchased from C. F. Boehringer and Söhne, Mannheim, Germany and was used without further purification. Cytochrome *c* and pyridine nucleotides were purchased from Sigma, (St. Louis, Mo., U.S.A.). Other reagents were purchased from Calbiochem (Los Angeles, Calif., U.S.A.).

RESULTS

Distribution of malate dehydrogenase among the particles

In Table I are shown data from a typical experiment, giving the volumes of the fractions collected as well as the approximate sucrose concentrations and appearance of the fractions collected by differential and isopycnic gradient centrifugation. It should be noted here that Fractions 3, 5, and 7 included the interface between layers of different sucrose concentration. At these interfaces were concentrated particles whose density was less than that of the next sucrose layer.

The distribution of malate dehydrogenase among the 4 fractions of the original

TABLE I

FRACTIONS FROM DIFFERENTIAL AND SUCROSE DENSITY GRADIENT CENTRIFUGATION

Volumes for the whole chloroplast, broken chloroplast, and mitochondrial fractions are those after resuspension of the pellets in the grinding medium.

Fraction	*Sucrose (M)*	*Vol. (ml)*	*Comments*
Differential centrifugation			
Original		125	118 ml used for centrifugation
Chloroplast		8.1	
Peroxisome and broken chloroplast		10.0	4.0 ml used for gradient
Mitochondrial		7.8	
Supernatant		104	
Isopycnic gradient centrifugation			
1	2.5	3.4	Clear
2	2.5–2.3	6.7	Clear
3	2.3–1.8	3.1	Peroxisomes at interface, yellow-brown band
4	1.8	5.3	Clear
5	1.8–1.5	4.4	Whole chloroplasts and mitochondria at interface, green band
6	1.5	9.6	Clear
7	1.5–1.3	7.3	Chloroplast fragments at interface, green band
8	1.3	9.9	Chloroplast fragments, green band
9	1.3–0.5	4.2	Supernatant, clear yellow

TABLE II

MALATE DEHYDROGENASE AND OTHER ASSAYS FOR CHARACTERIZING FRACTIONS FROM DIFFERENTIAL CENTRIFUGATION

Specific activity as nmoles · min^{-1} · mg^{-1} protein.

	Original	*Whole chloroplast*	*Peroxisome and broken chloroplast*	*Mitochondria*	*Supernatant*
Malate dehydrogenase					
Percent		6.9	41.3	3.7	48.1
Specific activity	560	326	1216	521	540
Glycolate oxidase					
Percent		4.7	31.2	2.5	61.3
Specific activity	21	10.7	44.4	16.9	33.2
Cytochrome *c* oxidase					
Percent		8.0	46.2	16.5	28.4
Specific activity	25	15.3	54.9	93.9	13.3
Chlorophyll					
Percent		25.6	63.6	5.5	5.3
$mg \cdot ml^{-1}$	0.58	2.3	4.6	0.5	0.0
Protein					
Percent		14.0	22.4	4.7	58.5
$mg \cdot ml^{-1}$	19.5	33.5	43.6	11.7	11.0

homogenate after differential centrifugation is compared in Table II with other characteristics used to distinguish the various particles. Three points should be noted. (1) Although malate dehydrogenase is a known constituent of the tricarboxylic acid cycle of the mitochondrion, among the particulate fractions, malate dehydrogenase showed the highest specific activity in the peroxisome fraction. In contrast, cytochrome *c* oxidase, a mitochondrial marker, showed the highest specific activity in the mitochondrial fraction. This indicates that there must be another source of malate dehydrogenase activity beside the mitochondria in the particulate fractions. (2) Although 25% of the chlorophyll was found in the whole chloroplast fraction, less than 7% of the malate dehydrogenase activity was found in this fraction. This low activity can be attributed to the presence of peroxisomes and mitochondria in this fraction, as indicated by the percentages of glycolate oxidase and cytochrome *c* oxidase. Further it should be noted that the specific activity of the malate dehydrogenase was greater in the original sap than in the chloroplast fraction. (3) The percentage of malate dehydrogenase in each fraction ranged between those of the marker enzymes glycolate oxidase and cytochrome *c* oxidase. If an enzyme is located in two different organelles, the percentage of this enzyme in each fraction should be close to an average of the percentages for the other organelle markers, assuming similar specific activities for the isozymes of the particular enzyme.

Fig. 1 shows the distribution of the enzyme activities, chlorophyll and protein in the isopycnic sucrose density gradient. Glycolate oxidase served as the marker enzyme for peroxisomes which were mainly in Fraction 3. Cytochrome *c* oxidase, the marker enzyme for mitochondria, was mainly in Fraction 5 and partly in Fraction 7.

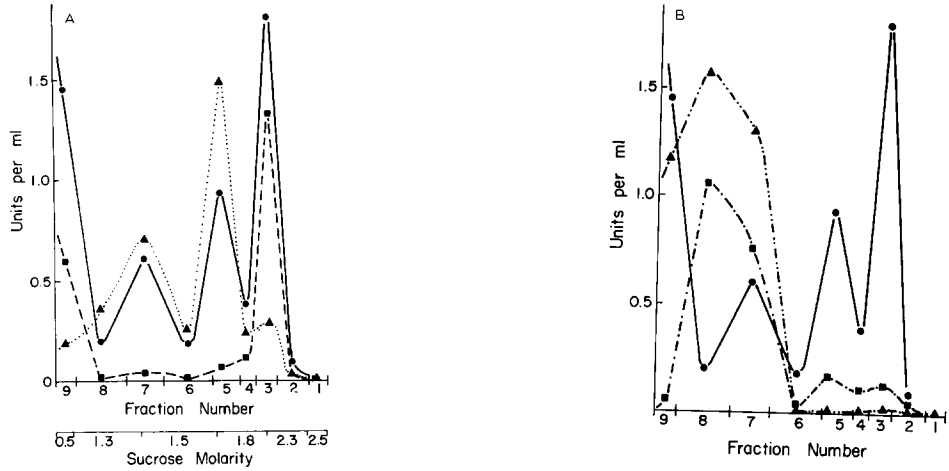


Fig. 1. Distribution of malate dehydrogenase and other marker enzymes among fractions from isopycnic sucrose gradient centrifugation. Malate dehydrogenase, glycolate oxidase and cytochrome *c* oxidase units expressed as μmoles substrate transformed per min per ml at 25°. Protein and chlorophyll expressed as mg/ml. ●—●, malate dehydrogenase; ■— — —■, glycolate oxidase; ▲· · · · ·▲, cytochrome *c* oxidase; ▲—· ·—▲, protein ($^1/_5$ scale); ■—·—■, chlorophyll.

Most of the chloroplasts, as indicated by chlorophyll, were in Fractions 7 and 8. Similar results were obtained with sunflower, morning glory, wheat, tobacco and perilla. Some of these data have been presented elsewhere[15]. The trimodal nature of the malate dehydrogenase distribution is evident. The two particulate malate dehydrogenases were in the peroxisomes and mitochondria and not in the chloroplasts. Two mitochondrial peaks, as measured by cytochrome *c* oxidase activity, occur because of the presence of interfaces in the gradient. A significant portion of the total malate dehydrogenase activity on the gradient was found in Fraction 9, the gradient supernatant fraction. Whether this activity arose from leakage of the peroxisomes and/or mitochondria or from actual soluble enzyme could not be ascertained. In earlier work with glycolate oxidase and glyoxylate reductase, it was noted that the ratio of the percent activity found in particulate fractions to that found in the supernatant fraction was similar for all three enzymes[14]. This suggested that the enzymes were present initially in the same particle and were released to the same extent upon isopycnic centrifugation. Thus, at least part of the malate dehydrogenase activity in the gradient supernatant fraction may be accounted for by leakage from the peroxisomes.

Peroxisomal malate dehydrogenase properties

Since spinach leaves are readily available and give high yields of peroxisomes, this tissue was used to study the characteristics of the peroxisomal malate dehydrogenase. Peroxisomes isolated by isopycnic centrifugation were used for these experiments without further purification.

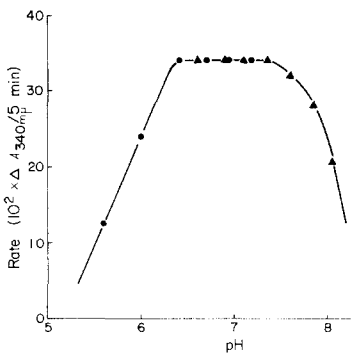
Latency. As in the cases of glycolate oxidase and glyoxylate reductase[14], the peroxisomal malate dehydrogenase activity was somewhat latent when the particle membrane was intact. When the nonionic detergent Triton X-100 was added to the

assay mixture, an increase in activity of about 30% was noted. To insure that all malate dehydrogenase activity would be measured, 0.01% Triton X-100 was routinely added in all assays.

pH dependence. Two zwitterionic buffers were used to minimize changes in ionic strength with variation of the pH. The pH curve (Fig. 2) is very similar to that shown for a malate dehydrogenase preparation made from acetone powders of spinach leaves by HIATT AND EVANS[19]. These authors found a rather broad pH optimum, with activity slightly increasing from pH 6.4 to 7.4. The use of sodium phosphate buffer in their study may account for this increase in activity with increasing pH, since the ionic strength of the medium would increase as the pH was raised and malate dehydrogenase activity is affected by ionic concentration.

Pyridine nucleotide specificity. The peroxisomal malate dehydrogenase is specific for NADH. At a reduced pyridine nucleotide concentration of 6 mM, the rate of NADPH oxidation was only 0.6% that of NADH.

Malate dehydrogenase activity in purified glyoxylate reductase. Since the enzyme NAD-glyoxylate reductase (EC 1.1.1.26) has been shown to be present in spinach peroxisomes, it was of importance to determine whether or not any of the malate dehydrogenase activity of the peroxisomes could be attributed to this enzyme. Consequently a crystalline preparation of glyoxylate reductase was tested in the normal assay for malate dehydrogenase activity. The rate of oxalacetate reduction in the preparation was 2.6% of that of glyoxylate. This low level might be attributed to the presence of contaminating amounts of malate dehydrogenase which has a very high turn-over number.



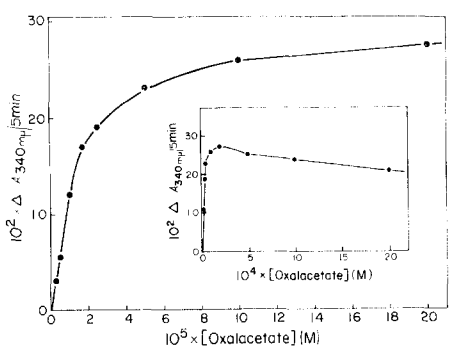


Fig. 2. pH dependency of peroxisomal malate dehydrogenase. Buffer pH adjusted by addition of KOH. Buffers: ●—●, 0.2 M *N*-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES); ▲—▲, 0.6 M HEPES. Other additions as described in the MATERIALS AND METHODS section.

Fig. 3. Effect of oxalacetate concentration on peroxisomal malate dehydrogenase activity. The standard assay procedure was used except that the oxalacetate concentration was varied as indicated. Peroxisomes purified by isopycnic centrifugation were used.

Kinetic behavior. In order to differentiate the various forms of malate dehydrogenase in the cell and to ascertain possible functions, the kinetic properties of the major malate dehydrogenase fractions were compared. The dependence of the reaction rate of the peroxisomal fraction (sucrose density gradient Fraction 3) on the oxalacetate concentration is given in Fig. 3. At oxalacetate concentrations greater than

$3 \cdot 10^{-4}$ M, some substrate inhibition was observed. From a Lineweaver–Burk plot the K_m (oxalacetate) was $1.4 \cdot 10^{-5}$ M, which is similar to that of $1.8 \cdot 10^{-5}$ M obtained by HIATT AND EVANS[19]. This suggests that the form isolated from acetone powders of spinach leaves was in fact the peroxisomal form.

A plot of the rate as a function of the substrate concentration for the mitochondrial fraction (sucrose density gradient Fraction 5) is shown in Fig. 4. Substrate inhibition began at an oxalacetate concentration of $7 \cdot 10^{-5}$ M and reached 50% at $2 \cdot 10^{-3}$ M. A Lineweaver–Burk plot gave a K_m (oxalacetate) of $5.7 \cdot 10^{-6}$ M for the mitochondrial malate dehydrogenase. The rate dependence of the supernatant fraction on the substrate concentration is shown in Fig. 5. A comparison of Figs. 3 and 4 with Fig. 5 gives the impression that the kinetic properties of the supernatant enzyme may be composite of the properties of the peroxisomal and mitochondrial forms, with the peroxisomes making the larger contribution.

Malate oxidation. Oxidation of L-malate by peroxisomal fractions could not be observed by following NAD^+ reduction at pH 7.4, probably because of the unfavorable equilibrium of the reaction. It was possible, however, to observe malate oxidation at pH 7.4, using 2,6-dichlorophenolindophenol and NAD^+ if diaphorase was added to the assay.

Salt effects. The effects of added salts in the assay were not studied extensively. Because of the similarities in properties of the peroxisomal malate dehydrogenase to the preparation described by HIATT AND EVANS[19], care was taken to exclude the addition of extraneous cations and anions in the assay medium. It was observed that the addition of 0.05 M NH_4Cl to the medium increased the rate of the reaction approx. 33%. It is interesting to note that another peroxisomal enzyme, glyoxylate reductase, also shows pronounced salt effects when hydroxypyruvate is utilized as the substrate[20].

Starch-gel electrophoresis. In Fig. 6 is shown a schematic representation of the patterns obtained upon starch-gel electrophoresis of the major malate dehydrogenase-containing fractions. The peroxisomal and mitochondrial forms were clearly differentiated by their mobility during electrophoresis, the mitochondrial form migrating

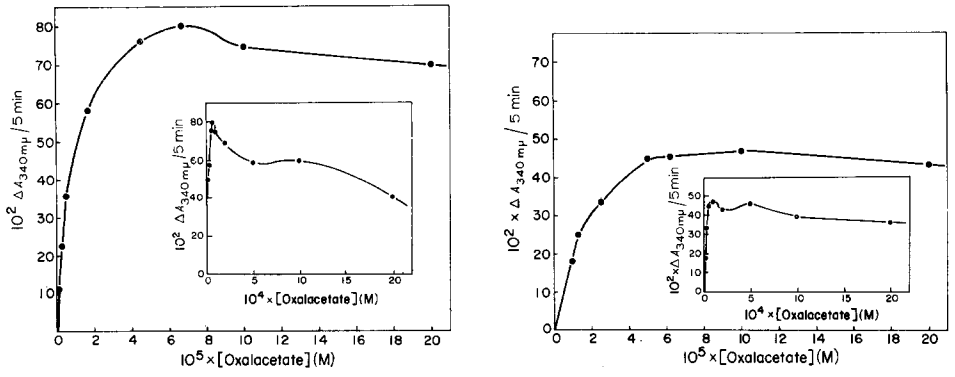


Fig. 4. Effect of oxalacetate concentration on mitochondrial malate dehydrogenase activity. Conditions as in Fig. 3 except that mitochondria purified by isopycnic centrifugation were used.

Fig. 5. Effect of oxalacetate concentration on supernatant malate dehydrogenase. Conditions as in Fig. 3 except that the supernatant from 37 000 × *g* centrifugation was used.

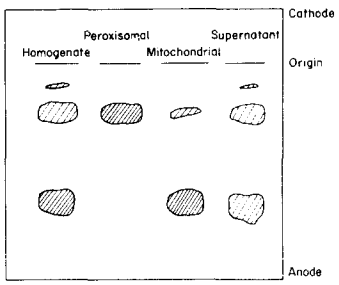


Fig. 6. Schematic representation of starch-gel electrophoresis patterns of the various malate dehydrogenases. Separation and staining techniques are described in the MATERIALS AND METHODS section.

the faster of the two. The supernatant fraction contained the peroxisomal and mitochondrial forms, with the peroxisomal form being the major component. In addition the supernatant contained a minor component which was not observed in particles. This component was not investigated further.

Comparison of specific activity of peroxisomal enzymes. Malate dehydrogenase, when measured in the direction of oxalacetate reduction, had a specific activity approx. 30 times greater than that of glycolate oxidase or glyoxylate reductase (Table III). Catalase had a still higher level of activity, which was about 25-fold greater than malate dehydrogenase.

TABLE III

SPECIFIC ACTIVITY OF PEROXISOMAL ENZYMES

Spinach peroxisomes isolated by isopycnic centrifugation were used without further purification.

Enzyme	*Specific activity* ($\mu moles \cdot min^{-1} \cdot mg^{-1}$ *protein*)
Glycolate oxidase	0.3–1.0
Glyoxylate reductase	0.3–1.0
Malate dehydrogenase	25–39
Catalase	500–600

DISCUSSION

In reports which locate malate dehydrogenase activity in chloroplasts, a common feature is the use of differential centrifugation for the isolation of chloroplast-enriched fractions. This use of only the sedimentation velocity properties of the cellular organelles for their purification has been shown by us to be unsatisfactory in the separation of chloroplasts and peroxisomes[14]. Thus with differential centrifugation of leaf homogenates at 1000 × *g*, one obtains a fraction enriched in both chloroplasts and peroxisomes. When this fraction is subjected to sucrose density gradient centrifugation at 1000 × *g* for short times, resolution of chloroplasts from peroxisomes is

still not possible, because the parameter used for separation is again sedimentation velocity. Apparently the sedimentation coefficients of the peroxisomes and chloroplasts are very similar. It is only through the use of another, distinct, physical property, such as buoyant density, that satisfactory separation of these particles can be achieved.

After differential centrifugation we have used isopycnic or equilibrium density centrifugation to obtain a separation of the chloroplasts, mitochondria and peroxisomes. A clear separation upon sucrose density gradient centrifugation resulted from the differences in the densities of the organelles. After equilibrium was attained (centrifugation at 100 000 $\times$ g for 3 h), the three types of particles were found in distinct bands centering about their own respective buoyant densities. Of the three organelles, the peroxisomes were the most dense, banding in the 1.95 M sucrose layer ($\varrho \cong 1.25$). The mitochondria banded in the 1.5 M sucrose ($\varrho \cong 1.20$) and the chloroplasts or chloroplast fragments banded in the 1.3 M layer ($\varrho \cong 1.17$). Collection of fractions of the appropriate volumes thus separated the organelles.

By these techniques malate dehydrogenase activity was found in the peroxisomal and mitochondrial fractions, whereas no malate dehydrogenase activity could be attributed to the chloroplasts. Chloroplasts which had been washed three times by differential centrifugation lacked malate dehydrogenase activity, although phosphoglycolate phosphatase, a chloroplast enzyme[13,21], remains with the chloroplasts (unpublished results of R. K. YAMAZAKI, S. L. VANDOR AND N. E. TOLBERT). We conclude that chloroplasts do not contain any malate dehydrogenase activity.

Similarities in pH behavior, pyridine nucleotide specificity, kinetic behavior and the effect of salts all suggest that the malate dehydrogenase activity isolated from acetone powders of spinach leaves by HIATT AND EVANS[19] was the peroxisomal form. This fact, together with the intracellular malate dehydrogenase distribution (Table II) and the comparison of the activities of the isozymes of the leaf homogenate after electrophoresis, suggests that the peroxisomal form contributes a major part of the total cellular malate dehydrogenase activity in the leaf.

The enzymes which have been found to date in leaf peroxisomes are glycolate oxidase, NAD-glyoxylate reductase, catalase, glutamate-glyoxylate transaminase (unpublished results of T. KISAKI AND N. E. TOLBERT) and malate dehydrogenase. All of these enzymes except malate dehydrogenase are associated with the direct metabolism of glycolate to glycine, *i.e.*, the glycolate pathway. Malate dehydrogenase is the only enzyme so far detected in the leaf peroxisomes which is not directly associated with glycolate metabolism.

As shown in Fig. 4, the spinach leaf mitochondrial malate dehydrogenase was noticeably inhibited by oxalacetate concentrations higher than $7 \cdot 10^{-5}$ M. This result is in accord with those found by other workers, using the mammalian mitochondrial enzyme, and has been interpreted as indicating that the physiological role of the mitochondrial enzyme is to provide for the oxidation of L-malate. On the other hand, the peroxisomal malate dehydrogenase was found to be much less sensitive to oxalacetate concentration. Similar differences in kinetic behavior between mitochondrial and 'soluble' malate dehydrogenases have been found in beef heart[22] and corn root tip[23]. In both cases, the nonmitochondrial form was thought to be catalyzing the reduction of oxalacetate to L-malate. Because of the similarities between these 'soluble' forms and the leaf peroxisomal malate dehydrogenase it is inviting to speculate that

the peroxisomal enzyme may likewise be utilized in the reduction of oxalacetate. Definite conclusions as to the function of the peroxisomal enzyme, however, must await further studies.

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