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L-MALATE : NADP OXIDOREDUCTASE (DECARBOXYLATING) FROM GERMINATING FLAX RUST UREDOSPORES*

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Abstract—L-Malate : NADP oxidoreductase (decarboxylating) (here called "malic" enzyme) from germinating flax rust uredospores was purified more than twenty-fold and partially characterized. This enzyme preparation was found to catalyze both the oxidative decarboxylation of L-malic acid to pyruvic acid at pH 7·6 and the decarboxylation of oxalacetic acid at pH 5. The oxidative decarboxylation of L-malate was found to specifically need NADP and required Mn^{2+} for maximum activity. Optimal pH for enzyme activity was 7·6. A Michaelis constant for L-malate of $1{\cdot}5 \times 10^{-4}$ *M* was found for this partially purified enzyme preparation. The stability and heat lability of the enzyme are reported in addition to the effect of a number of inhibitors.

INTRODUCTION

SEVERAL enzymes concerned with the intermediary metabolism of malate have recently been found to be quite active in cell-free extracts of germinating flax rust uredospores. These enzymes include malate synthase [L-malate glyoxylate-lyase (CoA-acetylating)] of the glyoxylate cycle[1] and malic dehydrogenase (L-malate : NAD oxidoreductase) of the Krebs cycle.[2] Another enzyme that has been found to be concerned with malate metabolism in other organisms is L-malate : NADP oxidoreductase (decarboxylating) (the "malic" enzyme).[3,4] This enzyme oxidatively decarboxylates L-malic acid to pyruvic acid and also catalyzes the decarboxylation of oxalacetic acid to pyruvic acid. It has recently been shown by Frear and Johnson[1] and Gottlieb and Caltrider[5] that malate may play an important role in the germinating uredospores of the flax and wheat rust fungi as an intermediate of the glyoxylate cycle enzyme system and in the synthesis of carbohydrates from large initial lipid reserves. Malate probably also functions as part of an active Krebs cycle system in these organisms. According to Staples,[6] malate is an early product in dark carbon dioxide fixation by bean and wheat rust uredospores.

During the investigation of the intermediary metabolism of germinating flax rust uredospores, it was noted that crude homogenates of germinated uredospores reduced NADP in the presence of added L-malate. These results appeared to be indicative of an active "malic" enzyme. This paper is a report of the purification and partial characterization of this enzyme from germinating flax rust uredospores.

RESULTS

Preparation of crude cell-free extracts

Lyophilized, germinated flax rust uredospores were homogenized with 5 ml of 0·05 *M* phosphate buffer, pH 7·0, per g of uredospores for 15 min in a glass Potter–Elvehjem tissue

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[1] D. S. FREAR and M. A. JOHNSON, *Biochim. Biophys. Acta* **47**, 419 (1961).
[2] K. W. DOCKTER and D. S. FREAR, *Proc. N. Dakota Acad. Sci.* **14**, 95 (1960).
[3] S. OCHOA, A. H. MEHLER and A. KORNBERG, *J. Biol. Chem.* **174**, 979 (1948).
[4] I HARARY, S. R. KOREY and S. OCHOA, *J. Biol. Chem.* **203**, 595 (1953).
[5] D. GOTTLIEB and P. G. CALTRIDER, *Phytopathology* **52**, 11 (1962), Abstract.
[6] R. C. STAPLES and L. H. WEINSTEIN, *Contrib. Boyce Thompson Inst.* **20**, 71 (1959).

homogenizer driven at 1000 rev/min. The remaining unruptured uredospores (usually a few non-germinated uredospores) and large uredospore and germ tube cell wall fragments were removed from this crude homogenate by centrifugation at $500 \times g$ for 5 min. The resulting whole spore and spore fragment pellet was resuspended and washed 3 times with 3 ml 0·05 *M* phosphate buffer, pH 7·0. The wash supernatants were combined with the original supernatant to make up the crude cell-free extract used for further enzyme purification.

Enzyme purification

The crude homogenate (Fraction 1) was centrifuged at $35{,}000 \times g$ for 30 min and the resulting supernatant (Fraction 2) was saturated to 35 per cent with solid ammonium sulfate. After centrifugation at $9000 \times g$ for 10 min, the supernatant (Fraction 3) was retained and brought to 75 per cent saturation with solid ammonium sulfate. The resultant pellet from further centrifugation at $9000 \times g$ was resuspended in 0·05 *M* phosphate buffer pH 7·0 (Fraction 4) and dialyzed 4 hr against 0·01 *M* phosphate buffer pH 7·0 with 3 changes of buffer. The supernatant which resulted from centrifugation of Fraction 4 at $9000 \times g$ was labelled Fraction 5. Fraction 5 was then adsorbed on calcium phosphate gel (24 mg/ml) using a 4 : 1 gel : protein ratio. Fraction 6 was obtained by gradient elution from the gel with 0·05 *M* and 0·10 *M* phosphate buffers pH 7·0. The results of a typical purification appear in Table 1.

TABLE 1. PURIFICATION OF FLAX RUST MALIC ENZYME

Fract. No.	Procedure	Vol. (ml)	Activity (units/ml)	Total (units)	Protein (mg/ml)	Specific activity (units/mg)	Yield (%)	Purification
1	Crude homogenate	30·5	40	1220	6·9	5·8	100	1
2	Supernatant after $35{,}000 \times g$ for 30 min	29·0	33	957	4·5	7·3	78·5	1·3
3	35% $(NH_4)_2SO_4$ supernatant	29·5	47	1385	3·9	12	113	2·1
4	35%–75% $(NH_4)_2SO_4$ pellet	11·7	114	1333	5·6	20	109	3·4
5	Fraction 4, dialyzed and centrifuged	13·5	90	1212	2·4	37·5	99	6·5
6	Gel eluate	5·1	44	224	0·33	133	18	23

Storage of the purified enzyme at 0° for two weeks resulted in a 70 per cent loss of activity. Heating the enzyme for 5 min at 50° resulted in 100 per cent loss of activity.

Effect of substrate and enzyme concentration

Data were obtained from the optical assay at 340 mμ which indicated the rate of reaction at various levels of enzyme and substrate concentration. The proportionality of activity versus enzyme concentration is depicted in Fig. 1. Fig. 2 is a Lineweaver–Burke plot of the L-malate requirement for enzyme activity. A Michaelis constant (K_m) of $1{\cdot}5 \times 10^{-4}$ *M* was found. This compares with values of 5×10^{-5} *M* reported for the pigeon liver enzyme[7] and 7×10^{-4} *M* reported for the wheat germ enzyme.[4]

[7] J. B. VEIGA SALLES and S. OCHOA, *J. Biol. Chem.* **187**, 899 (1950).

Metal and cofactor requirements

The purified enzyme was shown to be activated by Mn^{2+} as shown in Fig. 3. Activity of the enzyme in the presence of Mg^{2+} and Co^{2+} was found to be only 40 per cent as great as in the presence of equivalent amounts of Mn^{2+}. Some enzyme activity was found in the absence of added Mn^{2+}. Attempts were made to remove any Mn^{2+} bound to the enzyme by dialyzing the enzyme preparation against 0·01 *M* phosphate buffer pH 7·0 containing 0·001 *M* ethylenediaminetetra-acetic acid. This dialysis resulted in a substantial irreversible

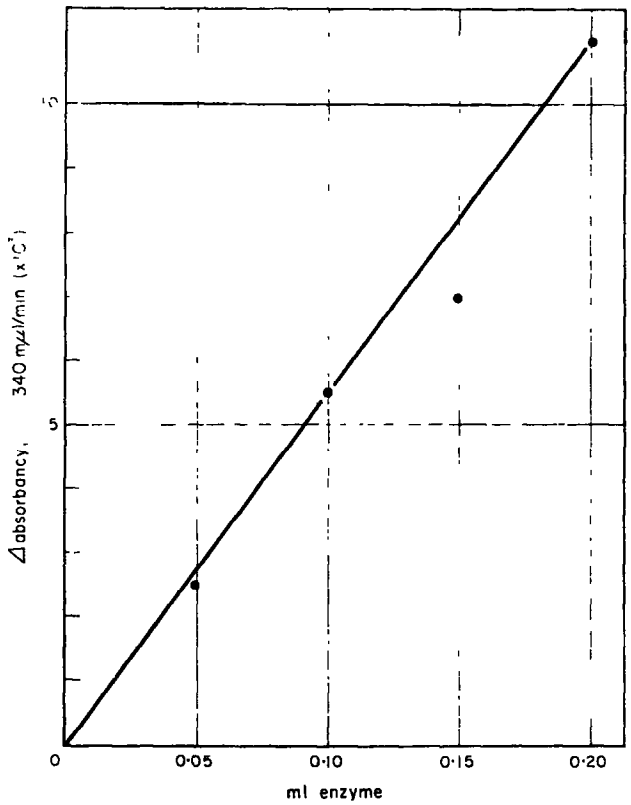

FIG. 1. EFFECT OF INCREASING ENZYME CONCENTRATION ON MALIC ENZYME ACTIVITY.
The reaction mixture consisted of 150 μmole *Tris* buffer (pH 7·0), 3 μmole $MnCl_2$, 0·52 μmole NADP, 15 μmole L-malate (pH 7·0), varying amounts of purified enzyme (0·33 mg protein/ml) and demineralized water to a total volume of 3·0 ml.

loss of enzyme activity. An approximate K_m value for Mn^{2+} was calculated from a Lineweaver–Burke plot of the added Mn^{2+} versus activity data depicted in Fig. 3. This K_m value was found to be $1{\cdot}1 \times 10^{-5}$ *M* and compares quite well with Mn^{2+} dissociation constants of $5{\cdot}0 \times 10^{-5}$ *M* and $2{\cdot}5 \times 10^{-5}$ *M* reported by Ochoa[8] for similar enzymes isolated from pigeon liver and wheat germ, respectively. A similarly determined K_m value for NADP was found to be $1{\cdot}1 \times 10^{-5}$ *M*. There was no enzyme activity in the absence of added NADP, and NAD could not substitute for NADP.

[8] S. OCHOA, *Methods in Enzymology*, Vol. I, p. 747. Academic Press, New York (1955).

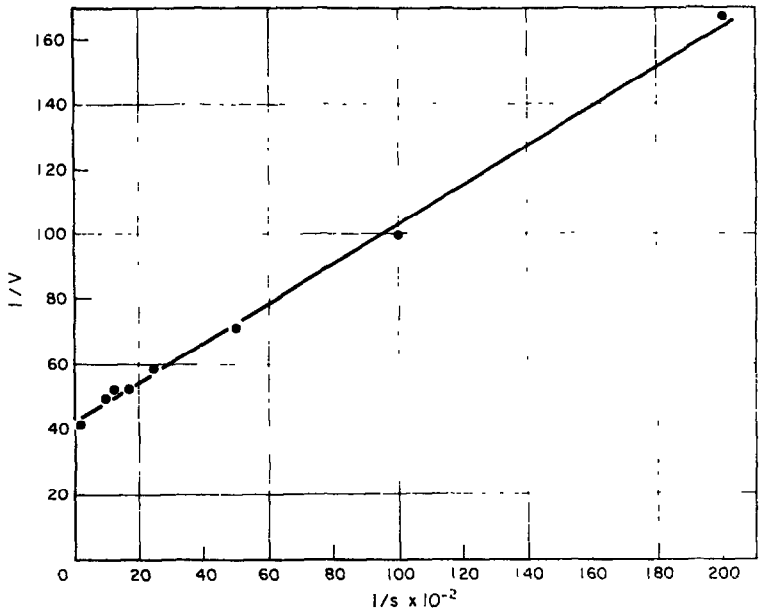

FIG. 2. SUBSTRATE REQUIREMENT OF MALIC ENZYME.
The reaction mixture consisted of 150 μmole *Tris* buffer (pH 7·0), 3 μmole $MnCl_2$, 0·52 μmole NADP, 0·1 ml of purified enzyme (0·33 mg protein/ml), varying amounts of L-malate (pH 7·0) and demineralized water to a total volume of 3·0 ml. Extrapolation of the straight line yields a K_m value of $1·5 \times 10^{-4}$ *M*.

TABLE 2. INHIBITION STUDIES OF FLAX RUST MALIC ENZYME

Inhibitor	Concentration ($M \times 10^5$)	Inhibition (%)
CN^-	100	100
	33	93
	17	43
	8·3	14
*$AsO_3^{\equiv}$	67	0
	33	0
*F^-	100	0
	33	0
N_3^-	100	25
	33	6
DDC	100	75
	33	0
PCMB	1	0
PCMPSA	1	13
Cu++	100	100
	33	31
Hg++	100	44
	0·5	19
OAA	100	55

* These anions show activation in crude preparations.

DDC = diethyldithiocarbamate
PCMB = *p*-chloromercuribenzoic acid
PCMPSA = *p*-chloromercuriphenylsulfonic acid
OAA = oxaloacetic acid

Effects of pH and inhibitors

The effect of a number of inhibitors upon the enzyme activity can be seen in Table 2.

The purified "malic" enzyme was found to have a rather sharp pH optimum at pH 7·6 as shown in Fig. 4. This corresponds quite well with the pH optima of 7·3 and 7·2 reported for the wheat germ[4] and the pigeon liver[9] "malic" enzymes respectively at the same substrate concentration (10^{-4} *M*).

Identification of reaction product

In addition to the standard assay procedure which demonstrated the production of $NADPH_2$, it was also possible to show the production of pyruvate as a consequence of the reaction. Pyruvate was identified as one of the reaction products through paper chromatography of the 2,4-dinitrophenylhydrazine derivatives isolated from the reaction mixture. The procedures used in these experiments were essentially those recommended by Krupka and Towers.[10] Alanine was identified by paper chromatography as the only electrolytic

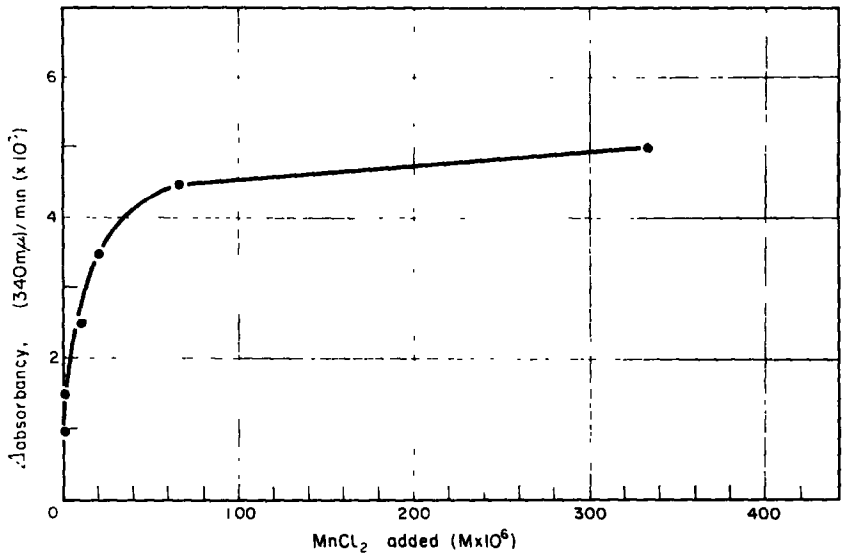

FIG. 3. ACTIVATION OF MALIC ENZYME BY Mn^{2+}.
The reaction mixture consisted of 150 μmole *Tris* buffer (pH 7·0), 0·52 μmole NADP, 0·1 ml purified enzyme (0·33 mg protein/ml), 15 μmole L-malate (pH 7·0), varying amounts of $MnCl_2$ and demineralized water to a total volume of 3·0 ml.

reduction product of the 2,4-dinitrophenylhydrazones produced in the above experiments. Radioisotope experiments with DL-3-^{14}C-malate also demonstrated the formation of labelled pyruvate. The formation of radioactive pyruvate was determined by autoradiography of paper chromatograms containing the 2,4-dinitrophenylhydrazone derivative isolated from the reaction mixture.

Oxaloacetate carboxyase activity

It can be seen from the results illustrated in Fig. 5 that oxaloacetate decarboxylase activity is present in the "malic" enzyme preparations. It is also apparent that NADP has

[9] W. J. RUTTER and H. A. LARDY, *J. Biol. Chem.* **233**, 374 (1958).
[10] R. M. KRUPKA and G. H. N. TOWERS, *Bot. Rev.* **25**, 108 (1959).

an inhibitory effect upon this activity of the enzyme. This inhibitory effect of NADP on oxaloacetate decarboxylase activity has also been noted by Kraemer *et al.*[11] with the wheat germ "malic" enzyme and is in direct contrast to the oxaloacetate decarboxylase activity of the enzyme from pigeon liver which requires NADP.[9] The converse of the NADP inhibition of oxaloacetate decarboxylation is the inhibition of oxidative malate decarboxylation by oxaloacetic acid as shown in Table 2.

Other enzymes present

The most active "malic" enzyme preparations obtained were assayed for the presence of a number of other enzymes which might still be present as contaminants. Standard optical assays were used in these experiments.[12] Assays were made for L-malate : NAD oxidoreductase; L_s-isocitrate : NAD oxidoreductase (decarboxylating); D-glucose-6-phosphate : NADP oxidoreductase, 6-phospho-D-gluconate : NAD oxidoreductase; and L-lactate : NAD oxidoreductase. The only enzymes of this group which were found to be active in this enzyme

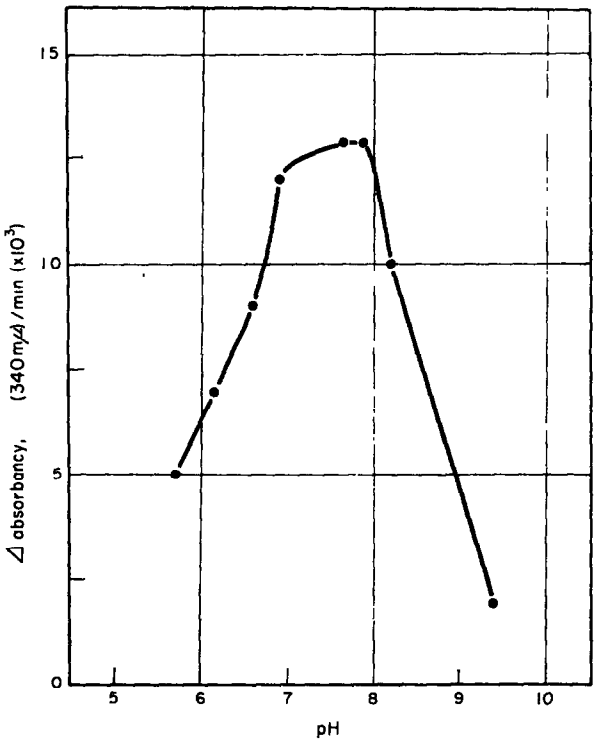

FIG. 4. OPTIMAL pH FOR MALIC ENZYME ACTIVITY.
The reaction mixture consisted of 150 μmole buffer (*Tris*–HCl, *Tris*–maleate, or glycine-NaOH), 0·52 μmole NADP, 3 μmole $MnCl_2$, 15 μmole L-malate (pH 7·0), 0·1 ml of purified enzyme (0·33 mg protein/ml) and demineralized water to a total volume of 3·0 ml.

preparation were the NAD-specific malic dehydrogenase (134 units/ml) and NADP-specific glucose-6-phosphate dehydrogenase (44 units/ml). In each case, a unit of enzyme is defined as the change in absorbancy at 340 mμ of 0·01 during the first minute of the reaction.

[11] L. M. KRAEMER, E. E. CONN and B. VENNESLAND, *J. Biol. Chem.* **188**, 583 (1951).
[12] S. P. COLOWICK and N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, New York (1955).

DISCUSSION

It may be noted from Table 1 that there is a slight rise to greater than 100 per cent yield in Fractions 3 and 4. Since this increase in yield was reproducible it is possibly due to the removal of an endogenous inhibitor. During the purification reported, the total protein drops from an initial 210 mg to less than 2 mg.

Pigeon liver "malic" enzyme is little affected by added arsenite[9] and the inhibitor studies with the flax rust enzyme shown in Table 2 tend to confirm these observations. Concentrations of 10^{-5} *M* *p*-chloromercuribenzoic acid and *p*-chloromercuriphenylsulfonic acid had

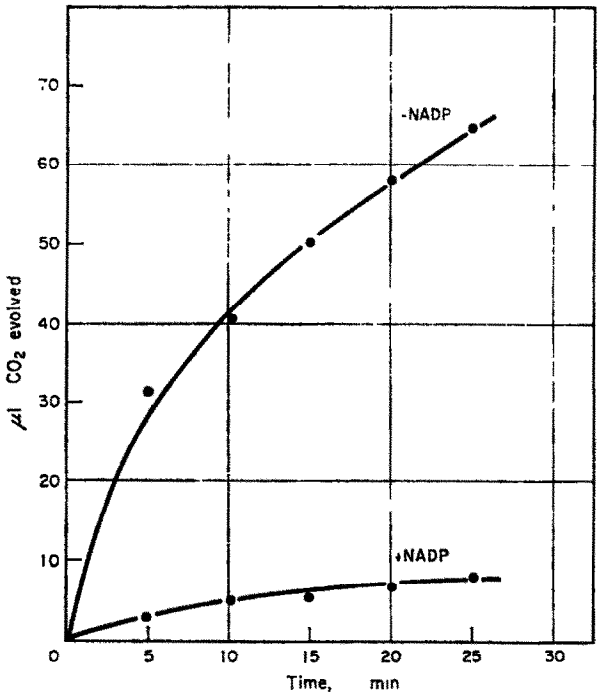

FIG. 5. OXALOACETATE DECARBOXYLASE ACTIVITY OF THE MALIC ENZYME.
The reaction mixture (total volume of 1·0 ml) consisted of 100 μmole acetate buffer (pH 5·2), 10 μmole $MnCl_2$, 3 μmole NADP or water, 20 μmole fresh oxaloacetate and 0·3 ml of purified enzyme (0·33 mg protein/ml). The enzyme was placed in the sidearm of the Warburg flask and tipped in to start the reaction. The specific activity of this oxaloacetate decarboxylase preparation was 84.

little effect on the flax rust enzyme in contrast to their effect on the pigeon liver enzyme.[9] This difference in the effect of these mercaptide-forming mercurial reagents is probably due to the much higher specific activity of the pigeon liver enzyme and the inherent differences in the enzyme source materials. The complete inhibition by 10^{-3} *M* Cu^{2+} and diethyldithiocarbamate may be attributed to the formation of insoluble complexes observed at that concentration in each instance.

EXPERIMENTAL

Uredospore materials

Race no. 1 of flax rust uredospores [*Melampsora lini* (*Pers.*) *Lév.*] were harvested from infected Bison flax plants (*Linum usitatisimum L.*) grown in the greenhouse. Freshly

harvested uredospores were germinated by dusting them on 50 ml of demineralized water in 6 in. Petri dishes (approximately 75–100 mg/dish). Germination was generally better than 90 per cent and was allowed to proceed for 12 hr at 16°. The resulting germinated uredospore mats were filtered off at room temperature by vacuum filtration and washed with demineralized water. The washed, germinated uredospores were then lyophilized and stored at −17° in vacuum-sealed glass vials until used for enzyme extraction.

General

All steps in the extraction and purification of "malic" enzyme were carried out at 0–4°. All centrifugation steps were carried out in a Spinco Model L preparative ultracentrifuge with a No. 30 rotor. A Beckman Model DK-2 ratio recording spectrophotometer equipped with a time drive attachment and a temperature-regulated cell holder maintained at 25±1° was used for all spectrophotometric measurements. Matched silica cells with 1·0 cm light paths were used in all spectrophotometric experiments. Protein was determined by the method of Lowry, *et al.*,[13] using crystalline bovine serum albumin as the standard. All pH measurements were made with a Beckman Model G pH meter.

Chemicals

Oxidized nicotinamide–adenine dinucleotide (98–100 per cent purity) and nicotinamide–adenine dinucleotide phosphate (95–100 per cent purity) were obtained from Sigma Chemical Company. The 3-^{14}C-labelled DL-malic acid (99 per cent radiopurity) was obtained from Calbiochem Corp. L-malic acid was obtained from Nutritional Biochemicals Corp. and was recrystallized once. The calcium phosphate gel was prepared according to the method of Keilin and Hartree.[14] All other reagents were commercial preparations of analytical reagent grade.

Enzyme assay procedures

The "malic" enzyme assay used was essentially that of Ochoa.[9] This assay is based on the measurement of the rate of reduction of NADP at 340 mμ in the presence of enzyme and excess L-malate. The reaction mixture contained 150 μmole *Tris* [2-amino-2-(hydroxymethyl)-1,3-propanediol] buffer pH 7·0, 3·0 μmole $MnCl_2$, 0·52 μmole NADP, 15 μmole L-malate, enzyme and demineralized water to a final volume of 3·0 ml. The reaction was initiated by the addition of substrate. Readings of the change in absorbancy were made against a blank containing all components except substrate. The increase in absorbancy at 340 mμ during the first minute after the start of the reaction was used to calculate enzyme activity. Under these conditions, the rate of NADP reduction was linear for at least 2 min with up to 2·2 enzyme units. One unit of enzyme activity is defined as that amount of enzyme which causes an increase in absorbancy at 340 mμ of 0·01 per min under the above conditions.

Measurement of oxaloacetate decarboxylase activity in the "malic" enzyme preparations was accomplished in the Warburg apparatus according to the procedure of Ochoa.[9] The assay was carried out in approximately 6 ml Warburg vessels in an air atmosphere at 25°. In a typical experiment the following reactants were placed in the main compartments of the flasks: 0·1 ml of 1 *M* acetate buffer pH 5·2 (100 μmole), 0·1 ml of 0·1 *M* $MnCl_2$ (10 μmole),

[13] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[14] D. KEILIN and E. F. HARTREE, *Biochem. J.*, **49**, 88 (1951).

0·3 ml of 0·01 *M* NADP (3 μmole), and 0·2 ml of 0·1 *M* freshly prepared oxaloacetic acid (20 μmole). The 0·3 ml of enzyme was placed in the side-arm and tipped in to start the reaction. No carbon dioxide evolution was detected in the absence of substrate. Appropriate corrections were made for the non-enzymatic decarboxylation of oxaloacetic acid by substituting boiled enzyme for enzyme. Readings were made every 5 min after the initiation of the reaction. The pH's of the flask contents were determined at the end of each experiment. One unit of enzyme was defined as that amount causing an excess carbon dioxide evolution over the blank of 1 μl in 10 min when calculated for the second 5 min interval after the beginning of the reaction. Specific activity was expressed as units of enzyme per mg of protein.