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MALATE DEHYDROGENASE IN *ZEA MAYS*: PROPERTIES AND INHIBITION BY SULFITE

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SUMMARY

Sephadex fractionation of malate dehydrogenase from *Zea mays* yields two forms with molecular weights of $2.3 \cdot 10^5$–$2.4 \cdot 10^5$ and $4.6 \cdot 10^5$–$4.8 \cdot 10^5$, respectively. For pyruvate carboxylation, only the enzyme-bound Mn^{2+} is needed. For malate decarboxylation, the K_m with respect to extraneously added Mn^{2+} is 1.6 μM. Mn^{2+}, however, also regulates the affinity of the enzyme to its substrate: compared with Mn^{2+}-saturating conditions, at half-saturating ones, the K_m for malate is raised from 0.090 to 0.180 mM. The SO_3^{2-} inhibition with respect to Mn^{2+} is partially competitive. The inhibition pattern with respect to malate and Mn^{2+} reveals a close interrelation between the binding of both of these reactants and SO_3^{2-}.

Sulfite inhibits malate decarboxylation in a partially competitive way with respect to $NADP^+$. The inhibition of pyruvate carboxylation with respect to NADPH represents a mixed type.

In malate decarboxylation at Mn^{2+}-saturating conditions, SO_3^{2-} is acting partially competitively with respect to the substrate. Thus, it behaves like CO_2-saturated bicarbonate, which previously had been characterized as a partially competitive inhibitor of malate decarboxylation. In pyruvate carboxylation, SO_3^{2-} is inhibiting in a fully competitive manner as had been found in ribulose-diphosphate carboxylase and phosphoenolpyruvate carboxylase. This indicates that the competition of SO_3^{2-} for the CO_2 or HCO_3^- binding site represents a general feature.

INTRODUCTION

The malate formers among the plants with the C_4-dicarboxylic acid pathway of photosynthesis and the *Crassulacean* acid metabolism plants are characterized by a high activity of malate dehydrogenase (L-malate: NADP-oxidoreductase, decarboxylating, EC 1.1.1.40) [1]. Thus CO_2 is provided for its refixation by ribulose-diphosphate carboxylase [3]. The enzyme is inactive with NAD, but its activity depends on the presence of Mn^{2+} or Mg^{2+}, as does the NAD-dependent enzyme in mitochondria

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[4]. The malate dehydrogenase of *Bryophyllum*, which was studied in detail by Brandon and Van Boekel-Mol [5] is markedly different from that of *Zea mays* [6] with respect to molecular weight, pH optimum, and affinity towards Mn^{2+}. The backward reaction (reductive carboxylation of pyruvate) takes place only at relatively high CO_2 tension or at high HCO_3^- concentrations. However, CO_2-saturated bicarbonate inhibits the decarboxylation of malate [2]. This is not due to the backward reaction but due to a partially competitive inhibition of the enzyme (see [7]) with respect to malate [2]. Even CO_2 (or HCO_3^-) does not take part in the reaction, each is bound at its specific site and thus reduces the affinity of the enzyme towards the substrate.

In ribulose-diphosphate carboxylase as well as in phosphoenolpyruvate carboxylase, SO_3^{2-}, which is the sulfite species prevailing at pH 7.8–8, is a fully competitive inhibitor with respect to HCO_3^- [8, 9]. Thereby, the increased affinity of phosphoenolpyruvate carboxylase towards HCO_3^- is correlated with a higher K_i value for SO_3^{2-}. Consequently the action of SO_3^{2-} on malate dehydrogenase seems to be of interest from a double point of view: firstly, with respect to its key position in the C_4- and *Crassulasean* acid metabolism plants for the evaluation of their sensitivity towards SO_3^{2-}, and secondly to prove whether HCO_3^- and SO_3^{2-} are not only competing in cases of fully competitive inhibition but also whether they are interchangeable in the case of partial competitive inhibition with respect to malate.

MATERIAL AND METHODS

Fully developed, 20–40-cm-long leaves were used from plants which had been grown in the period from May to October in the green-house at 20–30 °C and under natural light conditions. The activity in the crude homogenate of these plants was 250–300 μmoles NADP/mg chlorophyll/h, whereas, material grown from October to April showed much less activity (<100 μmoles NADP/mg chlorophyll/h), despite additional illumination.

Extraction, fractionation, and separation were carried out according to Johnson and Hatch [6]. For the dissolution of the $(NH_4)_2SO_4$ precipitate and for the elution of the Sephadex column, however, 50 mM Tris buffer at pH 8 plus dithioerythritol without Mn^{2+} or EDTA was used. The Sephadex fractions had a volume of 0.65 ml. For kinetic studies, those fractions were pooled which showed at least 30% of the maximum activity. If stored at 4 °C no loss was observed in the enzyme eluted from the column (see [5]) for a period of 3 weeks.

In the direction of oxidative decarboxylation, if not otherwise indicated, the assay medium contained: 2.7 ml 50 mM Tris buffer, (pH 8), 3 mM L-malate, 0.1 mM $NADP^+$ and 0.1 mM $MnCl_2$. The total volume was 3 ml. $\Delta\varepsilon$ at 340 nm was registered automatically every 20 s by a Leitz double-beam spectrophotometer. In the direction of reductive carboxylation, if not otherwise indicated, the assay medium contained: 0.2 ml 50 mM Tris buffer (pH 7.4), 16.6 mM pyruvate, 0.3 mM NADPH, 41.6 mM $NaH^{14}CO_3$ (5 μCi) and 3.33 mM $MnCl_2$. The total volume was 0.3 ml. After 5 min of incubation at 28 °C the reaction was stopped by the addition of 0.1 ml 80% acetic acid. Aliquots were counted with dioxan–butyl-PBD–naphthalin in the liquid-scintillation counter. Malate is the only $^{14}CO_2$ fixation product from this (see [10]). The omission of pyruvate resulted in a fixation rate of $< 4\%$.

The chlorophyll determination was done according to Arnon [11] and the

protein determination according to Warburg and Christian or Lowry (see [12]). For the calibration of the Sephadex column, "Combithek" calibration proteins (Boehringer) were used. Sephadex G-200 superfine was from Pharmacia, $NADP^+$ and NADPH from Boehringer, and L-malate and pyruvate from Sigma. All other chemicals were of analytical grade from Merck. $NaH^{14}CO_3$ was purchased from the Radiochemical Centre, Amersham. For all experiments only redistilled ion-exchange water was used.

RESULTS

(1) Activity and linearity of the reaction

Whereas in the crude extract the rate of malate decarboxylation was 8–9 μmoles NADP/mg protein/h and showed a lag phase of 60–90 s, the Sephadex-fractionated enzyme showed an activity of 400 μmoles NADP/mg protein/h. In both directions it was linear with respect to time and to enzyme concentration.

The pH optimum for pyruvate carboxylation was found at 7.4 (see [1]), and for malate decarboxylation at the malate concentration used, it was at pH 8 (see [6]). At substrate-saturating conditions, the relative activity of decarboxylation/carboxylation was 3:1. The enzyme did not react with NAD^+ or NADH, respectively.

(2) Molecular weight

During Sephadex chromatography, in agreement with the findings of Johnson and Hatch [6], the enzyme emerged shortly after the void volume. At their respective pH optimum and at substrate-saturating conditions, the activity in both directions shows a maximum, which corresponds to a mol. wt of $3.3 \cdot 10^5$ (Figs 1a and 1b). However, with respect to decarboxylation, a shoulder is already indicated. Pyruvate carboxylation, measured at pH 8, makes it obvious that the enzyme is composed of two molecular weight forms. This is also demonstrated by the omission of Mn^{2+} in the

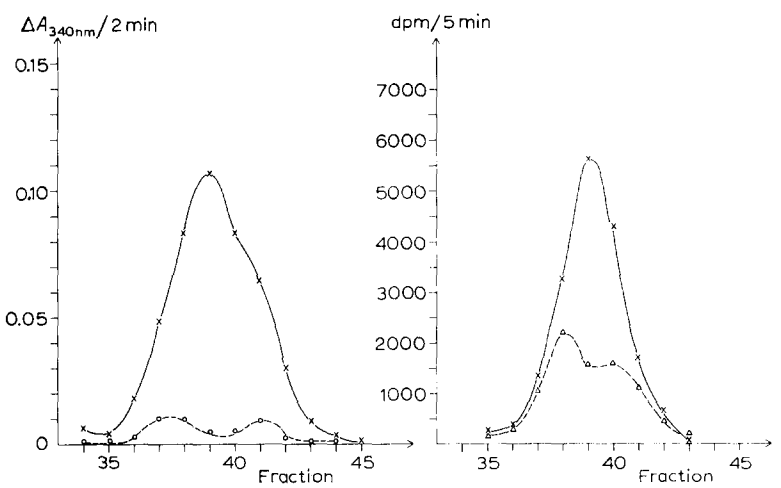

Fig. 1. (a) Malate decarboxylation in the Sephadex fractions. ×—×, at Mn^{2+}-saturating conditions; ○---○, without Mn^{2+}. (b) Pyruvate carboxylation in the Sephadex fractions. ×—×, at pH 7.4; △---△, at pH 8.0.

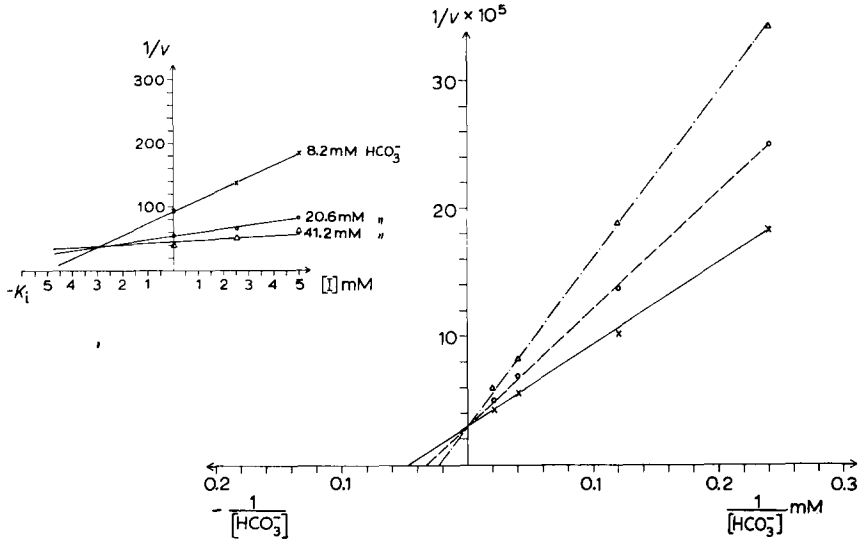

Fig. 2. Inhibition of pyruvate carboxylation by SO_3^{2-} with respect to HCO_3^-; Lineweaver–Burk plot. ×—×, without S_3O^{2-}; ○---○, 2.5 mM SO_3^{2-}; △-·-·△, 5 mM SO_3^{2-}.

decarboxylation reaction where, simultaneously, the activity is strongly reduced. The maxima correspond to mol. wts of $2.3 \cdot 10^5$–$2.4 \cdot 10^5$ and $4.6 \cdot 10^5$–$4.8 \cdot 10^5$, respectively.

(3) The Michaelis constants and the action of SO_3^{2-}

(a) HCO_3^-. In the direction of pyruvate carboxylation the K_m for HCO_3^- is 22 mM. SO_3^{2-} inhibits competitively (Fig. 2) with a K_i of 3 mM SO_3^{2-}. The plot of $\frac{1}{v}/[I]$ (see [7]) indicates a fully competitive inhibition; that is HCO_3^- and SO_3^{2-} are bound at the same site of the enzyme.

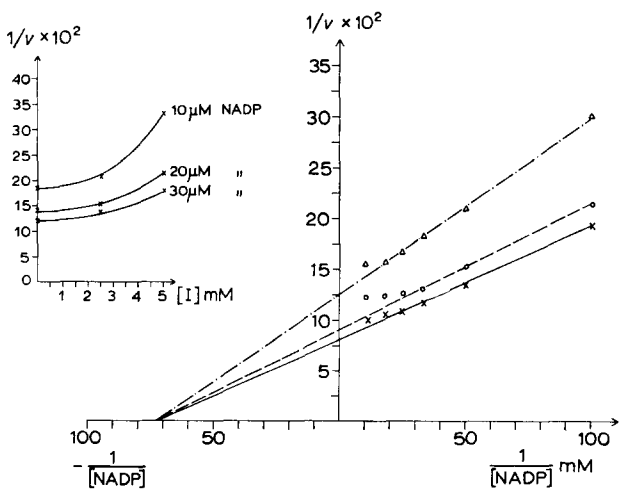

Fig. 3. Inhibition of malate decarboxylation by SO_3^{2-} with respect to $NADP^+$; Lineweaver–Burk plot. For legends, see Fig. 2.

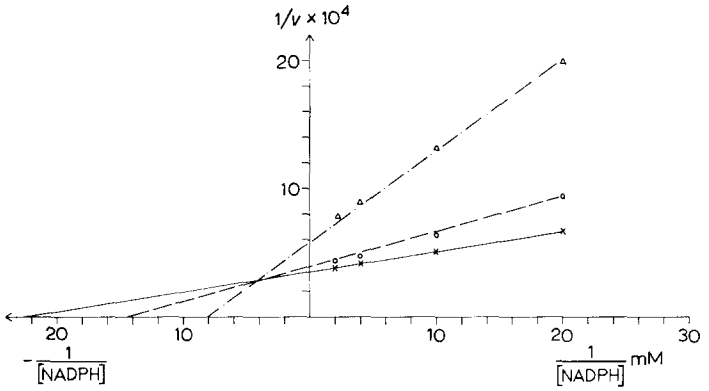

Fig. 4. Inhibition of pyruvate carboxylation by SO_3^{2-} with respect to NADPH; Lineweaver–Burk plot. For legends, see Fig. 2.

(b) NADP⁺ and NADPH. For malate decarboxylation the K_m of $NADP^+$ is 0.015 mM. The Lineweaver–Burk plot indicates a non-competitive inhibition; however, since the plot $\frac{1}{v}/[I]$ is not linear, a partially non-competitive inhibition is proven. The K_i can be calculated for 5 mM SO_3^{2-}.

In the direction of pyruvate carboxylation the K_m for NADPH is 0.045 mM. With SO_3^{2-}, a mixed type of inhibition is represented (Fig. 4).

(c) Malate and pyruvate. For malate decarboxylation, the K_m of malate is 0.090 mM. However, this is only true for saturating conditions with respect to Mn^{2+}.

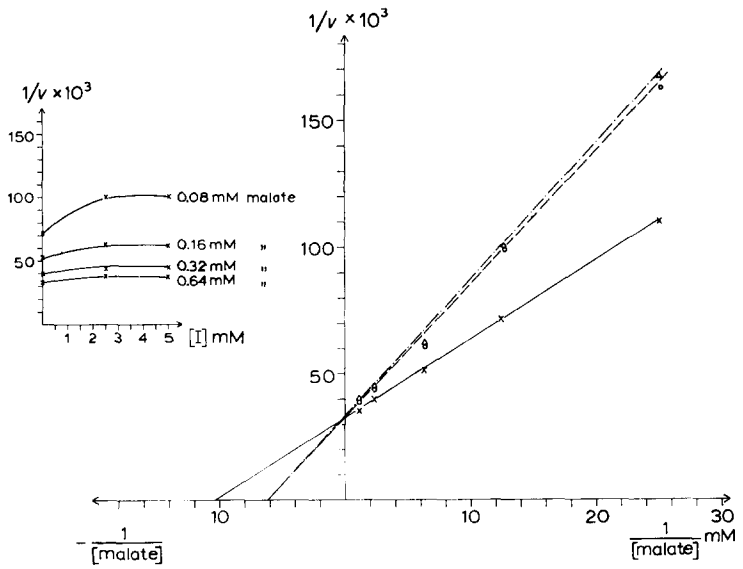

Fig. 5. Inhibition of malate decarboxylation by SO_3^{2-} with respect to malate at Mn^{2+}-saturating conditions; Lineweaver–Burk plots. For legends, see Fig. 2.

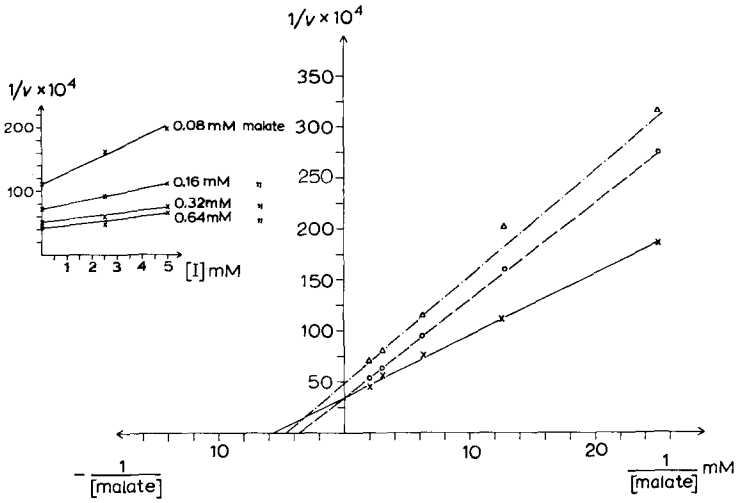

Fig. 6. Inhibition of malate decarboxylation by SO_3^{2-} with respect to malate at Mn^{2+} half-saturating conditions; Lineweaver–Burk plot. For legends, see Fig. 2.

At Mn^{2+}-half-saturating conditions (see Section 3d), the K_m rises to 0.180 mM. Moreover, Mn^{2+} strongly influences the inhibitory action of SO_3^{2-}. At saturating conditions, a partially competitive type of inhibition with respect to malate is shown which does not rise with the increase of the SO_3^{2-} concentration from 2.5 to 5 mM (Fig. 5). At 2 μM Mn^{2+}, the inhibition is of fully competitive type (Fig. 6), and an increase of the SO_3^{2-} concentration up to 5 mM also reduces the maximum velocity. Thus, at low

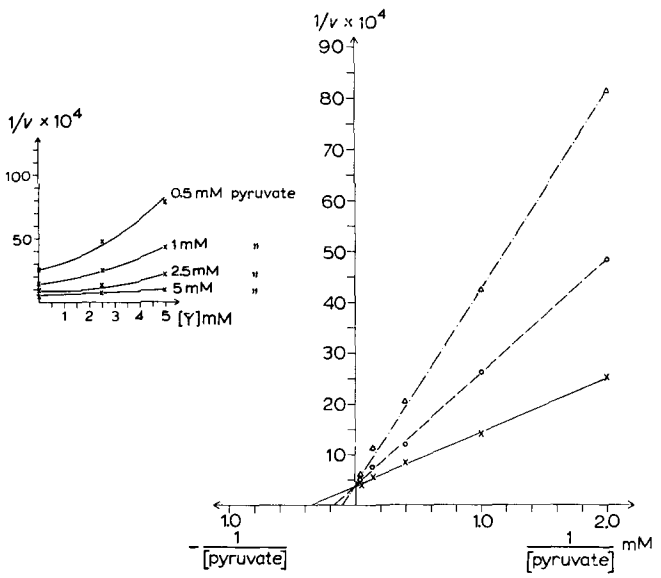

Fig. 7. Inhibition of pyruvate carboxylation by SO_3^{2-} with respect to pyruvate; Lineweaver–Burk plot. For legends, see Fig. 2.

Mn^{2+} levels, the concentration of the inhibitor not only changes the quantity but also the type of inhibition and a general K_i cannot be established.

For the carboxylation reaction the K_m of pyruvate is 3 mM. SO_3^{2-} inhibits partially competitively (Fig. 7). Obviously, since no additional Mn^{2+} is needed for the carboxylation reaction (see Section 3d), the type of inhibition with respect to the substrate is the same as it is for the decarboxylation at Mn^{2+}-saturating conditions. The K_i is 2.1–2.5 mM SO_3^{2-}.

(d) Manganese. It is evident that the decarboxylation of malate increases with the increasing concentration of Mn^{2+}. The K_m is 1.8 μM. However, Figs 8a and 8b show, that the saturation of the enzyme with Mn^{2+} moreover regulates its affinity to ma-

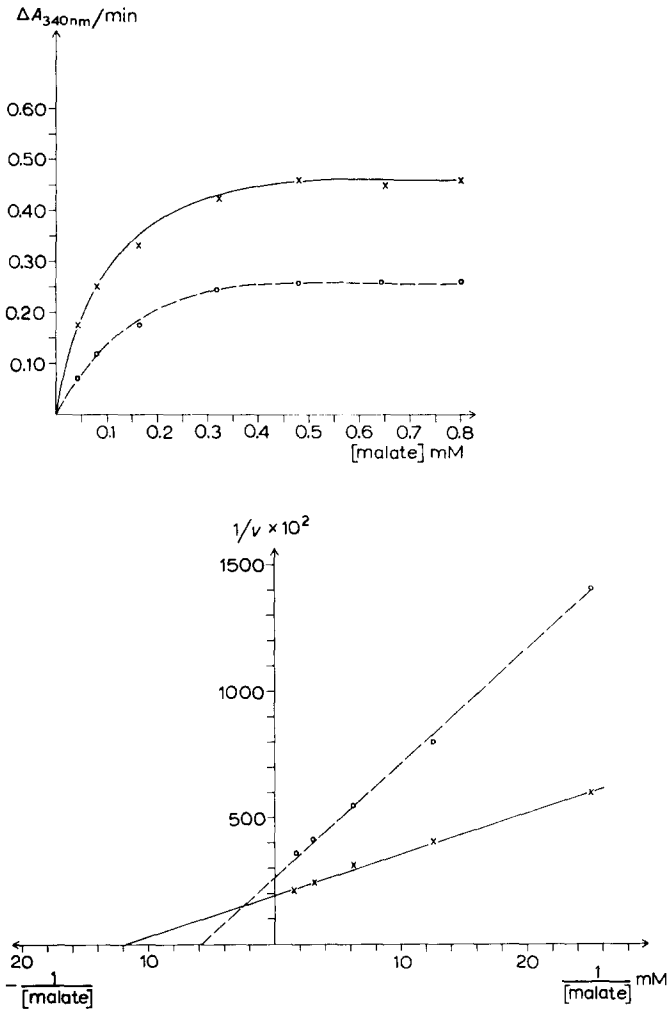

Fig. 8. (a) Malate decarboxylation with respect to malate in the presence of 100 μM Mn^{2+} (×—×) and 2 μM Mn^{2+} (○---○). (b) Lineweaver–Burk plot of (a).

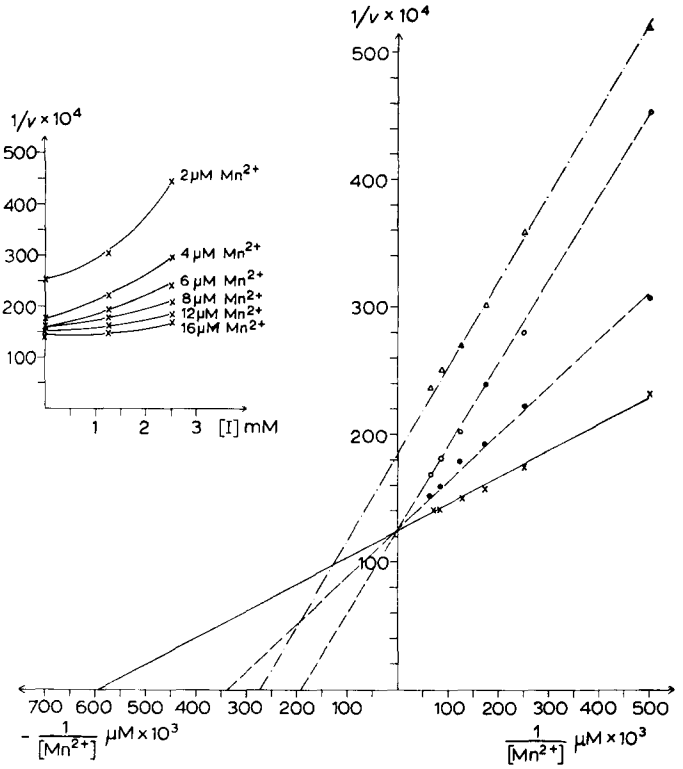

Fig. 9. Inhibition of malate decarboxylation by SO_3^{2-} with respect to Mn^{2+}, Lineweaver-Burk plot. ×—×, without SO_3^{2-}; ●---●, 1.25 mM SO_3^{2-}; ○---○, 2.5 mM SO_3^{2-}; △-·-·-△, 5 mM SO_3^{2-}.

late; at Mn^{2+}-half-saturating conditions, the K_m for malate is doubled (see Section3c).

1.25 and 2.5 mM SO_3^{2-} inhibit partially competitively (Fig. 9). As with respect to malate, at 5 mM SO_3^{2-}, the maximum velocity is also reduced and thus a general K_i cannot be given. Disregarding the qualitative change at 5 mM SO_3^{2-}, the K_i would be as low as 1.9 mM SO_3^{2-}, indicating a very sensitive point of attack.

TABLE I

ACTION OF Mn^{2+} ON THE ACTIVITY OF MALATE DEHYDROGENASE IN THE DIRECTION OF PYRUVATE CARBOXYLATION AT SATURATING CONDITIONS WITH RESPECT TO ALL OTHER REACTANTS

	Amount of dpm	
	3.33 mM Mn^{2+} in the assay system	Without Mn^{2+} in the assay system
Activity of the pooled Sephadex fractions	2873	2610
2-h dialysis against 50 mM Tris buffer (pH 7.4)	183	192
Incubation with 2 μM Mn^{2+} after dialysis	193	169
Addition of 0.5 mM EDTA in the assay system	77	65

With the pooled Sephadex fractions, pyruvate carboxylation takes place without addition of Mn^{2+} in the assay medium (Table I). Dialysis drastically reduces the activity which is not restored by subsequent incubation with Mn^{2+}. Addition of EDTA completely inactivates the enzyme. This indicates that for the decarboxylation, extraneous Mn^{2+} is needed which concomitantly regulates the enzyme's affinity for the substrate; whereas in the carboxylation reaction only the enzyme-bound Mn^{2+}, which can be removed by dialysis, takes part. SO_3^{2-} also competes for the enzyme-bound Mn^{2+} as it does for the additional one in the decarboxylation reaction. As shown in Fig. 10, the inhibition is largely compensated for with an increasing concentration of Mn^{2+}.

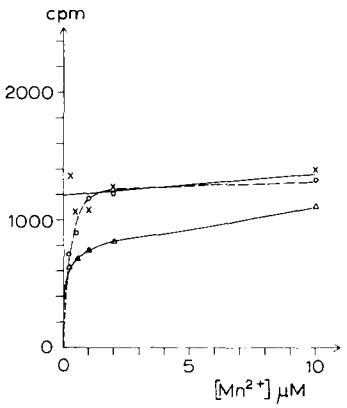

Fig. 10. Inhibition of pyruvate carboxylation by SO_3^{2-} with respect to Mn^{2+}. ×—×, without SO_3^{2-}; ○---○, 2.5 mM SO_3^{2-}; △—△, 5 mM SO_3^{2-}.

DISCUSSION

As mentioned above, it is indicated that the malic enzyme in leaves of *Zea mays* [6] differs from that in *Crassulaceans* [5] or in mango fruit [10] with respect to molecular weight and kinetics. Both the latter ones are similar to the enzyme from liver (see [5]). Pyruvate carboxylation at pH 8 as well as malate decarboxylation without the addition of Mn^{2+} reveals that the malate dehydrogenase, characterized here, has the same molecular weight ($2.3 \cdot 10^5$–$2.4 \cdot 10^5$) as that from Crassulaceans, and besides, that it can form its dimer (mol. wt $4.6 \cdot 10^5$–$4.8 \cdot 10^5$). At least during the isolation procedure which was employed, the monomeric and the dimeric form coexist in such a way, that both show the same activity in malate decarboxylation at Mn^{2+}-saturating conditions as well as in pyruvate carboxylation at pH 7.4. Thus we get an apparent mol. wt of $3.3 \cdot 10^5$–$3.5 \cdot 10^5$. Because we have no information about the subunits in plant malic enzyme, the characterization of both molecular weight forms needs further investigation.

For pyruvate carboxylation, as in malate dehydrogenase from *Pseudomonas aeruginosa* [13] only the enzyme-bound Mn^{2+} is needed. In the decarboxylation reaction, however, extraneous Mn^{2+} not only controls the activity, but moreover regulates the affinity of the enzyme to the substrate. The enhanced affinity at Mn^{2+}-saturating conditions entails that an increase of sulfite concentration beyond a certain level has no effect, whereas, at half-saturating conditions, further SO_3^{2-} is bound to the enzyme.

Possibly this occurs at another site, since the affinity of the enzyme is not reduced further, rather the maximum velocity is decreased. The interrelation between SO_3^{2-} binding on the one side and the binding of Mn^{2+} and malate on the other may have steric reasons. In a number of metalloenzymes, the role of the metal in the maintainance of the enzyme structure is well established (see [14]): in the case of plant malate dehydrogenase it awaits further elucidation.

The very low K_i of 1.9 mM SO_3^{2-} with respect to Mn^{2+} demonstrates a sensitive factor for SO_2 damage. An optimal supply with Mn^{2+} is surely indicated as a preventive factor.

The central question, as to whether competition between HCO_3^- and SO_3^{2-} occurs also in the case of malate dehydrogenase, can be answered in the affirmative. In pyruvate carboxylation, a fully competitive inhibition with respect to HCO_3^- takes place: the inhibitor is bound at the same site, EI does not react further and is unable to bind the substrate. Thus, the inhibition increases with increasing inhibitor concentration and $(1/v)/[I]$ is linear. This is also valid for the data obtained with the ribulose-diphosphate carboxylase and the phosphoenolpyruvate carboxylase [8, 9]. The K_i of 3 mM SO_3^{2-} indicates, that in the malic enzyme the HCO_3^- is as easily replaced by SO_3^{2-} as it is in the ribulose-diphosphate carboxylase (K_i = 3 mM SO_3^{2-}); whereas in phosphoenolpyruvate carboxylase, it is 27 mM SO_3^{2-}.

As mentioned above, during malate decarboxylation a partially competitive inhibition by CO_2-saturated bicarbonate with respect to malate is taking place (l.c. [2]). At Mn^{2+} saturation conditions this is also true for SO_3^{2-}, indicating that both inhibitors bind at the same site of the enzyme apart from the substrate binding site. Thus, HCO_3^- and SO_3^{2-} are competing for the same site not only when HCO_3^- takes part in the reaction as a substrate, but also when it reduces the affinity of the enzyme to malate. Consequently, the competition of SO_3^{2-} for the CO_2 or HCO_3^- binding site may be of general importance and may represent the first point of attack of SO_2 in plant metabolism.

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