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EFFECT OF SULPHITE ON PHOSPHOENOLPYRUVATE CARBOXYLASE AND MALATE FORMATION IN EXTRACTS OF *ZEA MAYS*

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Key Word Index—*Zea mays*; Gramineae; maize leaves; phosphoenolpyruvate carboxylase; malate dehydrogenase; SO_3^{2-} inhibition.

Abstract—The effect of SO_3^{2-} on the activity of PEP-carboxylase and on subsequent malate formation has been studied in leaf extracts of *Zea mays*. PEP-carboxylase was assayed by incorporation of $H^{14}CO_3^-$ into oxaloacetate dinitrophenylhydrazone and by a spectrophotometric method. In contrast to ribulose diphosphate carboxylase, PEP-carboxylase was not inhibited by 10 mM SO_3^{2-} with respect to PEP. As was the case with ribulose diphosphate carboxylase, the activity of PEP-carboxylase was inhibited non-competitively with respect to Mg^{2+}. However, the K_i value (84·5 mM) was found to be very high. With respect to HCO_3^-, like ribulose diphosphate carboxylase, PEP-carboxylase was inhibited competitively, but the K_i value (27 mM SO_3^{2-}) increased by about the same factor ($\times 9$) as the K_m (0·5 mM HCO_3^-) is decreased. This indicates that the replacement of HCO_3^- by SO_3^{2-}, common to both enzymes, is facilitated by decreasing the affinity of the enzyme for HCO_3^-. At substrate saturating conditions malate formation by the combined action of PEP-carboxylase and endogenous NADH-dependent malate dehydrogenase in leaf extracts was not inhibited by 10 mM SO_3^{2-}. Although the malate dehydrogenase is inhibited at this SO_3^{2-} concentration to about 85%, malate formation is unaffected, as PEP-carboxylase is the rate limiting step its turnover rate being only about 8% of NADH-dependent malate dehydrogenase.

INTRODUCTION

IN AN earlier paper[1] on the effect of sulphur-dioxide on photosynthetic CO_2 uptake it was shown that SO_3^{2-} causes a non-competitive inhibition of ribulose diphosphate carboxylase with respect to ribulose-1,5-diphosphate and Mg^{2+}, but acts competitively with respect to HCO_3^-. This implies that in the living cell the inhibitory action of SO_2 is highly dependent on the CO_2 (or HCO_3^-) concentration at the site of CO_2 fixation. Thus it is of interest to know whether in plants operating with the C_4 pathway of photosynthesis increased CO_2 concentration in the bundle sheath cells (see Ref. 2) may result in an evolutionary advantage with respect to SO_2 resistance. The effect of SO_3^{2-} on phosphoenolpyruvate-carboxylase (PEP-carboxylase, E.C. 4.1.1.31), preceding the refixation by ribulose diphosphate carboxylase, however, must not impair this advantage. Further it seemed of interest to ascertain whether in PEP-carboxylase SO_3^{2-} is acting in the same way by replacing CO_2 and thus causing competitive inhibition, and whether the increased affinity of PEP-carboxylase (K_m between 0·25–0·45 mM HCO_3^-;[2]) compared with that of ribulose diphosphate carboxylase (2·5–30 mM HCO_3^-;[3]) raises the K_i value of SO_3^{2-}. As yet we have no

[1] ZIEGLER, I. (1972) *Planta (Berl.)*, **103**, 155.
[2] HATCH, M. D. and SLACK, C. R. (1970) *Ann. Rev. Plant Physiol.* **21**, 141.
[3] KAWASHIMA, N. and WILDNER, S. G. (1970) *Ann. Rev. Plant Physiol.* **21**, 325.

information on the action of SO_3^{2-} itself on PEP-carboxylase; only α-hydroxypyridine methane sulfonate and glyoxal bisulfite are reported to be inhibitory,[4] and the action of these compounds was considered as a model for SO_2 action.[5]

RESULTS

Incorporation of $H^{14}CO_3^-$ *into Oxaloacetate dinitrophenylhydrazone*

The bulk of PEP-carboxylase activity was found in Fraction I (Table 1). If NADH is omitted, the radiochromatogram of the PEP-carboxylase incubation product yields oxaloacetate dinitrophenylhydrazone as the only labelled compound. The R_f value of 0·45 is in good agreement with that found by Block *et al.*[6] Heating the product to 60° for 20 min, which causes decarboxylation and formation of pyruvate dinitrophenylhydrazone,[7] here also results in a complete loss of radioactivity. Thus the incorporation of $H^{14}CO_3^-$ corresponds to the activity of PEP-carboxylase. It is very close to that obtained by the usual spectrophotometric method, e.g. 13 μmol $H^{14}CO_3^-$/mg protein/hr and 14 μmol NADH/mg protein/hr respectively.

TABLE 1. ACTIVITY OF NADH-DEPENDENT MALATE DEHYDROGENASE AND PEP-CARBOXYLASE IN AMMONIUM SULFATE FRACTIONS OF *Zea mays*

Ammonium sulfate saturation %	NADH-dependent malate dehydrogenase (μmol NADH/mg protein/hr)	PEP-carboxylase + NADH-dependent malate dehydrogenase (μmol NADH/mg protein/hr)
40–60 Fraction I	43·5	11·0
60–75 Fraction II	232	6·9
40–75 Fraction I + II	139	9·8

In substrate saturating conditions, SO_3^{1-} up to a concentration as high as 10 mM has no inhibitory effect on PEP-carboxylase. Also at low concentrations of PEP (K_m value 0·16–0·20 mM) SO_3^{2-} exerts no inhibitory action. With varying $H^{14}CO_3^-$ (Fig. 1) a competitive type of inhibition is seen. The low inhibitory effect of SO_3^{2-} is expressed by its K_i value which is as high as 26–28 mM. K_m was found to be 0·5 mM HCO_3^-. Towards Mg^{2+}, SO_3^{2-} exerts a non-competitive type of inhibition (Fig. 2). Its extremely low sensitivity is indicated by the K_i value of 84·5 mM; K_m for Mg^{2+} is 0·48 mM.

Incorporation of $H^{14}CO_3^-$ *into Malate*

Mitochondrial malate dehydrogenase (E.C. 1.1.1.37) is known to be strongly inhibited by SO_3^{2-},[8] and the same is true for the NADH- and the NADPH-dependent malate dehydrogenase of maize.[9] However, when NADH is added to the incubation medium,

[4] OSMOND, C. B. and AVADHANI, P. N. (1970) *Plant Physiol.* **45,** 228.
[5] LÜTTGE, U., OSMOND, C. B., BALL, E., BRINCKMANN, E. and KINZE, G. (1972) *Plant Cell Physiol.* **13,** 505.
[6] BLOCK, R. J., DURRUM, E. L. and ZWEIG, G. (1958) *Paper Chromatography and Paper Electrophoresis,* Academic Press, New York.
[7] BANDURSKI, R. S. (1955) *J. Biol. Chem.* **217,** 137.
[8] PFLEIDERER, G., JECKEL, D. and WIELAND, TH. (1956) *Biochem. Z.* **187,** 328.
[9] ZIEGLER, I. unpublished.

$H^{14}CO_3^-$ incoporation into an acid-stable product shows little or no impairment by SO_3^{2-} if Fraction II or total active leaf protein (Fraction I + Fraction II) is used. The same is true for the spectrophotometric assay with endogenous malate dehydrogenase. Therefore, special attention was paid to the identification of the reaction product. However, malate was the only product, and the formation of aspartate by a transamination reaction can be ruled out. Fraction II which was used for this experiment, only resulted in a reduction of about 30% in $^{14}CO_2$ incorporation but did not change the pattern of fixation.

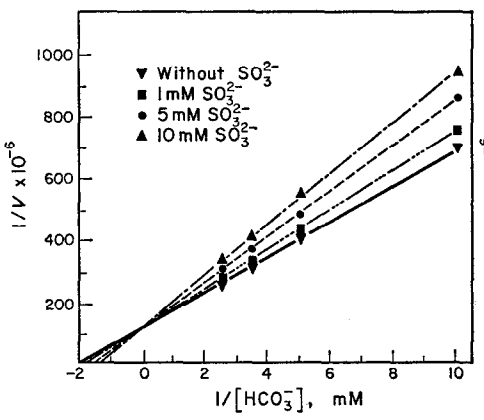

FIG. 1. INCORPORATION OF $H^{14}CO_3^-$ INTO OXALOACETATE DINITROPHENYLHYDRAZONE BY FRACTION I AT VARIOUS CONCENTRATIONS OF HCO_3^-. LINEWEAVER–BURK PLOT SHOWING COMPETITIVE TYPE OF INHIBITION.

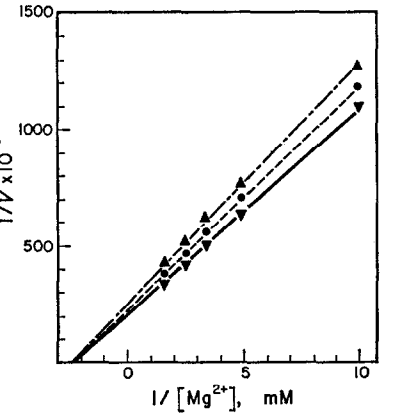

FIG. 2. INCORPORATION OF $H^{14}CO_3^-$ INTO OXALOACETATE DINITROPHENYLHYDRAZONE BY FRACTION I AT VARIOUS CONCENTRATIONS OF Mg^{2+}. LINEWEAVER–BURK PLOT SHOWING A NON-COMPETITIVE TYPE OF INHIBITION.

Table 1 shows that PEP-carboxylation is the rate limiting step not only in Fraction II and in total active leaf protein, but even in Fraction I, which comprises the bulk of PEP-carboxylase activity. Moreover, the data show that even at 85% inhibition by 5 mM SO_3^{2-} enough malate dehydrogenase activity is spared in Fractions I and II and consequently even more in Fraction II above, to metabolize the oxaloacetate delivered by the PEP-carboxylase. In Fraction I, 85% inhibition of malate dehydrogenase also impairs malate formation.

DISCUSSION

The results show that ribulose diphosphate carboxylase[1] and PEP-carboxylase are qualitatively affected by SO_3^{2-} in the same way: both are inhibited towards Mg^{2+} in a non-competitive type of inhibition, whereas SO_3^{2-} acts competitively with respect to HCO_3^-. Moreover with both enzymes the K_i value for competitive inhibition is lower than that for the non-competitive one, indicating that replacement of HCO_3^- by SO_3^{2-} represents a more general feature of SO_3^{2-} action. Comparison of K_m and K_i values of both enzymes (K_m HCO_3^- for ribulose diphosphate carboxylase 4·2 mM, for PEP-carboxylase 0·5 mM; K_i SO_3^{2-} 3·0 and 26–28 mM respectively) reveals the striking fact that with respect to HCO_3^- the K_i value for SO_3^{2-} in PEP-carboxylase is increased by about the same factor (×9) as the K_m value for HCO_3^- in ribulose diphosphate carboxylase (× 8·4).

This shows that with increasing affinity for the substrate HCO_3^-, the replacement by SO_3^{2-} is rendered more difficult, and this results in decreased sensitivity towards SO_3^{2-}.

The experiments above show that the action of HSO_3^-/SO_3^{2-}, which at pH 7–7·9 in a solution of $Na_2S_2O_5$ or Na_2SO_3 are present in a ratio of about 1:9·8,[1] is different from the action of α-hydroxy-pyridinemethane sulfonate or glyoxal bisulfite. The latter compounds are reported to inhibit PEP-carboxylase in a competitive way with respect to PEP, and from the data given the K_i value seems to be as low as about 1 mM (see Fig. 4 in Ref 4). Therefore, the action of these compounds cannot serve as a model for SO_2 action.[5]

EXPERIMENTAL

Extraction and fractionation of enzymes. Fully developed leaves of *Zea mays* (minimum length of about 20 cm) were extracted[4] without Polyclar. After centrifugation at 30000 *g* for 20 min, the supernatant was fractionated with solid $(NH_4)_2SO_4$. The protein precipitated between 40 and 60% $(NH_4)_2SO_4$ saturation is designated as Fraction I, that between 60 and 75% as Fraction II. The ppts were dissolved in the extraction medium, and for storage the solution was made 3·2 M with respect to $(NH_4)_2SO_4$.

Enzyme assay. (a) On coupling with endogenous malate dehydrogenase, the oxidation of NADH was measured at 340 nm. In these studies the concentrations of the components used were as in.[10] Incorporation of $H^{14}CO_3^-$ into oxaloacetate was measured, thus avoiding inhibition of malate dehydrogenase[8] by SO_3^{2-}. The assay mixture, if not otherwise indicated, contained: Tris buffer pH 8·0, 25 μmol; Mg^{2+} 1 μmol; PEP 2 μmol; glutathione 2·5 μmol or dithiothreitol 1 μmol and $H^{14}CO_3^-$ 10 μmol (2 μCi) in a total vol. of 0·5 ml. The reaction was started by addition of PEP, run for 6 min at 28° and stopped by addition of 0·2 ml of cold 2,4-dinitrophenylhydrazone (satd. solution in 2 N HCl). Aliquots were streaked on Schleicher–Schüll glass fibre No. 6, dried by a cold air stream and counted in a scintillation counter with butyl-PBD/dioxan/naphthalin. For identification, the reaction product was purified[11] at 4° and co-chromatographed with 2,4-dinitrophenylhydrazone of oxaloacetate previously prepared, in *n*-BuOH–H_2O–EtOH (5:4:1) at 12°. Addition of NADH (1 μmol) to the assay mixture described above causes incorporation of $^{14}CO_2$ into an acid stable product. In these experiments the reaction was stopped by 80% HOAc (0·2 ml) or 2 N HCl (0·02 ml) per 0·5 ml assay mixture and dried at 80° before counting. For identification of the reaction product the samples were dissolved in 0·2 ml H_2O and to each 0·2 mg malate and aspartate added. Tris was removed by a column (5 × 50 mm) of Dowex 1 (formate form). After washing with H_2O, fractions (0·15 ml) eluted with 6 N formic acid which showed radioactivity were pooled and chromatographed on Whatman No. I paper with BuOH–HCOOH–H_2O (10:2:15), followed by cellulose TLC with EtOAc–HCOOH–H_2O (10:2:3). The co-chromatographed aspartate was located by ninhydrin-spray, and malate by bromocresol green spray. The malate spot was further identified by the enzymatic test with malate dehydrogenase and NAD.[12] The NADH-dependent malate dehydrogenase was assayed according to Ref. 10.

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