Phytochemistry, Vol. 22, No. 12, pp. 2661–2664, 1983.
Printed in Great Britain.

0031–9422/83 $3.00 + 0.00
Pergamon Press Ltd.

DIFFERENTIAL INHIBITION OF PHOSPHOENOL-PYRUVATE CARBOXYLASES BY 2,4-DICHLOROPHENOXYACETIC ACID AND TWO NEWLY SYNTHESIZED HERBICIDES

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(*Received* 18 *March* 1983)

Key Word Index—Phosphoenol-pyruvate carboxylase; C_4 plants; C_3 plants; inhibition; chlorophenoxy compounds; herbicides.

Abstract—The effect of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) and two new phenoxy compounds on the *in vitro* activity of plant leaf phosphoenol-pyruvate carboxylases has been examined. The C_4 leaf enzymes (0.66 nkat) were completely inhibited by these chemicals (4 mM). The activity of the sorghum leaf enzyme was partially recovered when either the effector glucose-6-phosphate or the substrate phosphoenol-pyruvate was added to the medium after the reaction had been started in the presence of the inhibitors. Glycine, another enzyme activator, did not reactivate the inhibited enzyme. C_3 plant PEP carboxylases were little affected by the three herbicides. An intermediary inhibition was shown to occur in C_3 Gramineae and etiolated sorghum leaves. Other enzyme activities of carbon and nitrogen metabolism were unaffected. The results allow discrimination between the metabolic type of plants. In this respect, the best response was obtained with the newly synthesized monochlorinated derivative.

INTRODUCTION

Phosphoenol-pyruvate carboxylase (EC 4.1.1.31) is a widely distributed enzyme among C_3, C_4 and CAM plants [1–3]. In C_3 plant leaves, the carboxylating activity is catalysed by isoenzymes differentially located in the cell [4, 5]. The enzyme is thought to be needed to perform an anaplerotic function [6] by incorporation of inorganic carbon on the substrate PEP to feed the Krebs cycle pools of oxaloacetate and malate.

C_4 and CAM plants are characterized by a specific form of PEP carboxylase exhibiting a very high level of enzyme activity (10- to 20-fold higher when compared to C_3 plants). The enzyme has a key role in primary carbon dioxide fixation during photosynthesis in C_4 plants and displays different functional and regulatory properties from those of C_3 plants [7–11]. Recently, Rao *et al.* [12] have demonstrated *in vitro* as well as *in vivo* the inhibition of the enzyme activity in non-succulent scrub species by the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T).

In the present work, we have studied the effect of 2,4-D and newly synthesized related compounds on PEP carboxylase activity in C_3 and C_4 plants. We present evidence that these chemicals inhibit very specifically the C_4 form of the enzyme.

RESULTS

Kinetics of 2,4-D inhibition on the C_4 form of PEP carboxylase from sorghum leaves

These investigations were performed with 0.66 nkat of sorghum enzyme that had been purified by DEAE-cellulose chromatography. Maximal inhibition was observed after 1 min of incubation with 2 mM 2,4-D at 30° (Fig. 1A). An incubation period of 2 min was chosen for all further experiments. No activity was observed in the presence of 4 mM 2,4-D (Fig. 1B). When 10 μmol of glucose-6-phosphate, a specific activator of the C_4 form of carboxylase [13], was added to the medium after the reaction had been started in the presence of either 3 or 0.5 mM 2,4-D, the enzyme activity was partially recovered. Compared to the control, the activation was much higher in the 3 mM inhibited medium than in the 0.5 mM assay (Table 1). This is indicative of a partial reversal of the inhibition by the sugar phosphate. By contrast, glycine, another specific activator of the C_4 form, increased the enzyme activity by the same order of magnitude in both the control and the inhibited assays (Table 1).

The different components of the reaction medium, namely NADH, $NaHCO_3$ and $MgCl_2$, had no effect on the inhibition. The addition of Mg^{2+}, up to 15 mM, did not result in a reactivation of the inhibited C_4 (or the C_3) enzyme. Only PEP brought about a partial removal of the inhibition (Fig. 1C). A 2.5-fold increase in the substrate led to a maximal recovery of 80 and 50% of the control for assays inhibited by 0.5 and 1 mM 2,4-D, respectively.

Differential effects of 2,4-D and the two new derivatives

When assayed on 0.8 nkat of enzyme, the two new phenoxy compounds (**2** and **3**) were as efficient inhibitors as 2,4-D (**1**). The free acids of **1–3** were as effective as the potassium salts. 2,4-D was taken as the reference inhibitor. In order to obtain accurate comparative data, the amount of inhibitor vs the amount of enzyme and PEP was kept constant at 4 mM inhibitor and 2 mM PEP per 0.8 nkat of enzyme.

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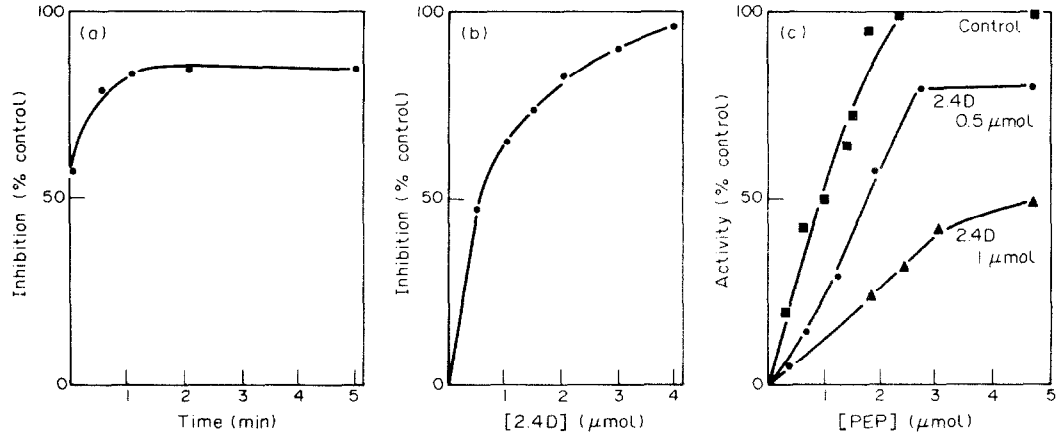


Fig. 1. Inhibition of green sorghum leaf PEP carboxylase (C_4 form) by 2,4-D. (A) Effect of incubation time. 0.66 nkat of enzyme (24 μg protein) was incubated for the time indicated with 2 μmol of the inhibitor at 30°. Reaction was initiated by the addition of the substrate PEP (2 μmol). (B) Effect of inhibitor concentration. Reaction was performed as in (A) except that the enzyme (0.66 nkat, 24 μg protein) was incubated for 2 min with different concentrations of inhibitor before the reaction was initiated by PEP. (C) Effect of PEP concentration. Reaction was performed as in (B), except that the enzyme was increased to 1 nkat, 36 μg protein, in 1 ml of medium, in the absence (■) or in the presence of 0.5 μmol 2,4-D (●) and 1 μmol 2,4-D (▲).

Table 1. Effect of glucose-6-phosphate and glycine on PEP carboxylase (C_4 form) from green sorghum leaves inhibited by 2,4-D

	Activity (% control)					
Assay	−G6P	+G6P	$\frac{+G6P}{-G6P}$	−Gly	+Gly	$\frac{+Gly}{-Gly}$
Control	100	205	2.0	100	242	2.4
2,4-D, 0.5 mM	54	140	2.6	66	155	2.3
2,4-D, 3 mM	16	92	5.7	17	44	2.6

The assays were performed as described in Fig. 1B with either 3 or 0.5 mM 2, 4–D. 10 μmol of glucose-6-phosphate or glycine was added 2 min after the start of the incubation in the presence of PEP.

By using these experimental conditions, it was found that PEP carboxylase of green leaves from C_4 plants was strongly inhibited (over 80%) by 2,4-D as well as by the two derivatives (Fig. 2). For the C_3 plants, 2,4-D and the derivative **3** showed a similar inhibition level of the enzyme activity, i.e. 50% in the case of Gramineae, only 20% in the case of non-Gramineae plants except in rice where it was far more active than 2,4-D. The derivative **2**, which was nearly as effective as the two other herbicides on C_4 plant enzymes, inhibited PEP carboxylases from C_3 Gramineae slightly (10–20%) but had no detectable effect on the carboxylase present in C_3 non-Gramineae.

Moreover, it was found that NAD-malate dehydrogenase which is coupled to PEP carboxylase in the assay, as well as other enzymes of nitrogen metabolism (glutamate oxoglutarate aminotransferase, glutamine synthetase) and carbon metabolism (malic enzyme, NADP-malate dehydrogenase), was practically insensitive to 2,4-D (data not shown).

DISCUSSION

The present work has been concerned with an examination of the influence of herbicides on PEP carboxylase activity. The enzyme extracted from green leaves of C_4 plants is inhibited by 2,4-D and other phenoxy compounds. This result is in good agreement with the previous report of Rao *et al.* [12], who found an inhibitory effect *in vitro* and *in vivo* of 2,4,5-T on the PEP carboxylase from non-succulent scrub species which displayed some characteristics of CAM plants.

With the sorghum enzyme (C_4 form), inhibition by 2,4-D is partially suppressed by the enzyme activator, glucose-6-phosphate, and by the substrate, PEP. This last result suggests that the phenoxy chemical can hinder the enzyme activity by competing with the substrate PEP at the catalytic site. However, as the recovery of activity is incomplete, it appears that the inhibition process is complicated and needs further examination. These results should be taken into account for *in vivo* studies using the herbicides.

Under the experimental conditions used in this work, phenoxy compounds are specific inhibitors of the C_4 form of the enzyme. In C_3 plants, the efficiency depends on the plants studied. They have almost no effect on the enzyme from non-Gramineae plants, whereas they inhibit the carboxylases present in Gramineae plants up to 50%. This

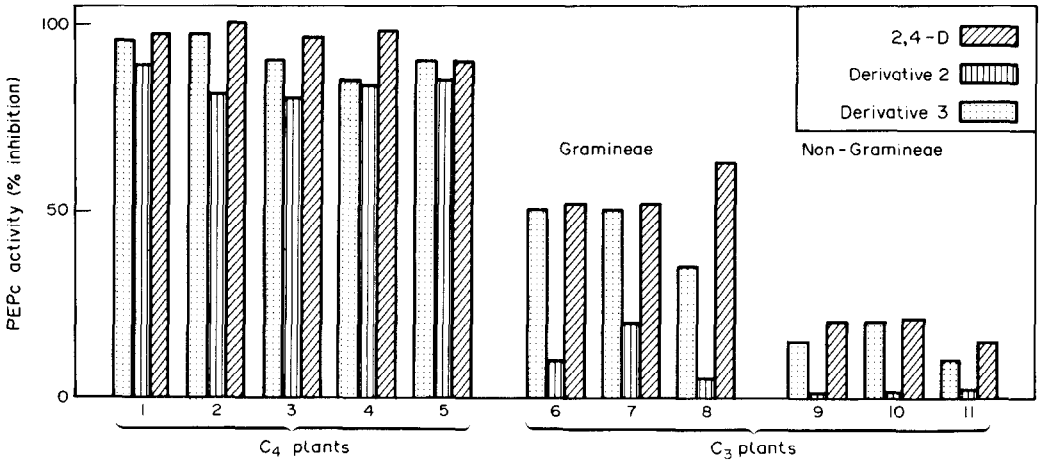


Fig. 2. Differential inhibition of plant PEP carboxylases by 2,4-D and two new compounds. The different plant PEP carboxylases were purified by chromatography on DEAE-cellulose. Standard assays were performed with 0.8 nkat of enzyme. After 2 min of incubation in the presence of 4 mM of inhibitor, the reaction was started with 2 μmol of PEP at 30°. The plants studied were (1) *Sorghum vulgare* (green), (2) *Pennisetum americanum*, (3) *Zea mays*, (4) *Panicum miliaceum*, (5) *Amaranthus retroflexus*, (6) *Sorghum vulgare* (etiolated), (7) *Triticum aestivum*, (8) *Oryza sativa*, (9) *Glycine max*, (10) *Pisum sativum*, (11) *Spinacia oleracea*.

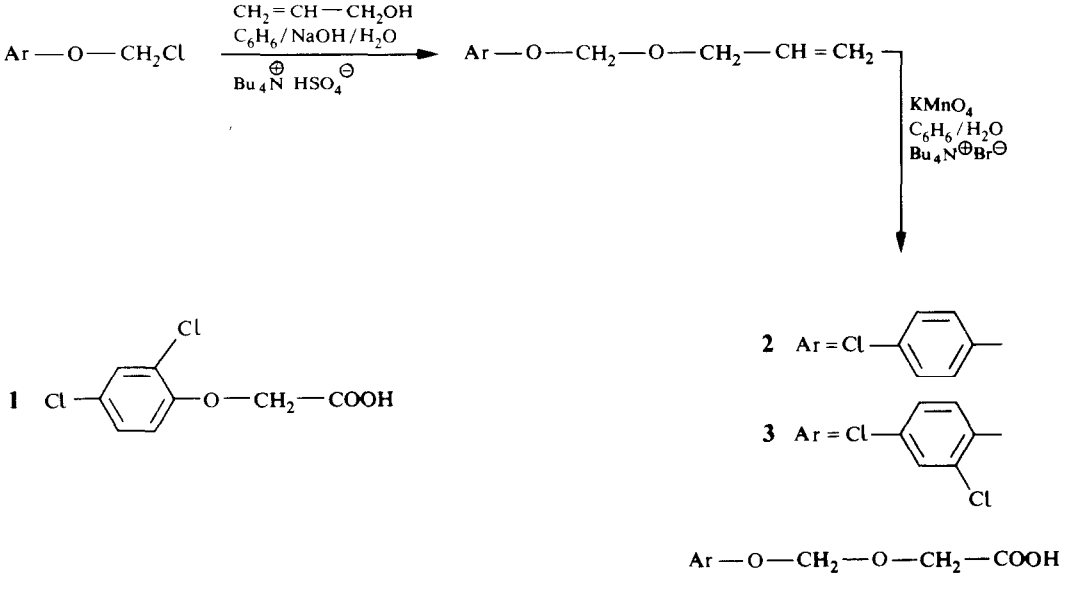
result provides evidence for functional similarities between the PEP carboxylases of Gramineae whatever their metabolic type. This finding has been corroborated by immunochemical studies (unpublished data).

The new derivative **3** exhibits the same levels of inhibition as 2,4-D, whereas the monochlorinated derivative (**2**) discriminates well between the C_3 and C_4 forms of the enzyme.

These data, if extended to other plants, could be very useful in allowing the estimation of the relative proportions of C_3 and C_4 forms of the carboxylases in the extracts and hence in distinguishing between the two types of plants. For example, sorghum leaves contain two PEP carboxylases: a C_3-like form, present in etiolated tissue; and a C_4 form which appears during greening. It was found that the former is quite insensitive to herbicide **2** (10% of inhibition) whereas the latter is strongly inhibited (90%). Furthermore, it has been shown that the activity of some other enzymes of nitrogen and carbon metabolism are not significantly affected by these herbicides.

EXPERIMENTAL

Plant material. Sorghum (*Sorghum vulgare* Pers. cv INRA 450) was germinated on vermiculite humidified by H_2O at 28° in darkness for 5 days. Half of the shoots were then illuminated



Scheme 1.

(500 W/m^2) by Grolux tubes, Sylvania, for 78 hr. All the other plants (C_4 plants: *Pennisetum americanum*, *Zea mays*, *Panicum miliaceum*, *Amaranthus retroflexus*; C_3 Gramineae: *Triticum aestivum*, *Oryza sativa*; C_3 non-Gramineae: *Glycine max*, *Pisum sativum*) were grown under similar conditions as described above. Spinach leaves (*Spinacia oleracea*) were bought fresh from the local market.

2,4-D and synthesis of the two new compounds. Commercial 2,4-D (Fluka) (**1**) was used as the reference product. Acetalic compounds **2** and **3** were prepared in good yields according to Scheme 1 (the details will be published elsewhere).

The new products **2** and **3** seemed to be active *per se*. Their efficiency did not appear to be similar to the herbicidal activity of ω-phenoxyalkanoic acids containing 3 or more carbon atoms which give phenoxyacetic acids by β-oxidation of the side chain [14].

1–**3** were dissolved in 100 mM Tris (Cl^-), pH 8.0, at a concn of 100 mM. The pH of these solns and of the inhibited assays were kept at 8.0.

Extraction and purification. The expts were performed at 2°. Leaf material (3 g fr. wt) was blended in 30 ml 25 mM Tris (Cl^-) buffer, pH 8, containing 100 mM mercaptoethanol, 5% glycerol and 300 mg Polyclar AT, by using a polytron homogenizer. The extract was filtered through gauze, de-aerated by N_2 and centrifuged at 50 000 *g* for 30 min. The supernatant was poured onto a column of DEAE-cellulose (1 × 10 cm), equilibrated in 25 mM Tris (Cl^-) buffer, pH 8, 14 mM mercaptoethanol and 5% glycerol. The column was rinsed with 40 ml equilibrium buffer. The enzyme was eluted from the column by increasing the concn from 0 to 400 mM of Tris (Cl^-) buffer, pH 8, containing 5% glycerol.

Enzyme assay. The standard assay mixture (1 ml) contained 100 mM Tris (Cl^-) buffer, pH 8, 1.8 mM PEP, 5 mM $MgCl_2$, 0.2 mM NADH and 16.67 nkat NAD-malate dehydrogenase. Oxidation of the cofactor was measured at 340 nm at 30°. Activity units are expressed in nkat.

Protein determination. Protein content was measured spectrophotometrically at 205 nm according to ref. [15].

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