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Short Communication

Inhibition of the β-Carboxylation Pathway of CO_2 Fixation by Bisulfite Compounds

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C. B. Osmond and P. N. Avadhani[1]
Research School of Biological Sciences, Australian National University, Box 475, Canberra City, 2601, Australia

Bisulfite compounds are well known as inhibitors of glycolate oxidase in green tissues of higher plants (2, 14). In an effort to understand the relation between low glycolate oxidase activity and high P-enolpyruvate carboxylase activity in plants with the C_4 dicarboxylic acid pathway of photosynthesis (7, 11), we have treated leaves of related species of *Atriplex* with these compounds. In this photosynthetic process, as well as during dark CO_2 fixation leading to acidification of *Sedum* leaves, we have found bisulfite compounds to be effective inhibitors of the P-enolpyruvate carboxylation system. This report provides evidence *in vivo* for this inhibition and describes the inhibition *in vitro* of P-enolpyruvate carboxylase (EC 4.1.1.31) and NADH malate dehydrogenase (EC 1.1.1.37).

The characteristics of photosynthetic CO_2 fixation in *Atriplex spongiosa*, F.v.M., a C_4-type plant, and *Atriplex hastata*, L., a conventional C_3 plant, have been described in detail elsewhere (9). Leaves taken from greenhouse-grown seedlings were allowed to fix $^{14}CO_2$ for 30 sec in a closed 50-ml container into which was injected 0.5 μmole of CO_2 containing 16 μc of ^{14}C in 10 ml of air. Light intensity was 250 $w \cdot m^{-2}$, and temperature was 28°. The leaves were killed in boiling 80% ethanol, and extracts were prepared and chromatographed as described previously (8). Prior to exposure to $^{14}CO_2$ leaves were placed with their petioles dipping in small vials of deionized water or solutions of α-HPMS[2] (Aldrich Chemical Co.). In other experiments glyoxal bisulfite (Fluka) and sodium bisulfite (British Drug Houses, MAR grade) were used. The bisulfite compounds were used as supplied and after recrystallization from aqueous ethanol. The inhibitors were absorbed with the transpiration stream, which was monitored by weighing the vials and leaves.

The leaves of *Sedum praealtum* A.D.C. used in these experiments showed a diurnal fluctuation of about 100 μeq/g, fresh wt, in acid content when the temperature varied between 30° during the day and 10° during the night. Leaves were removed at appropriate times during the diurnal cycle of dark CO_2 fixation, and the acid content was measured. Deacidification after a suitable dark period was measured in leaves kept under lamps in the laboratory (28°, 250 $w \cdot m^{-2}$). Samples of five leaves each (5 to 10 g, fresh wt) were injected at the base with about 0.5 ml of deionized water (controls) or the same volume of the appropriate concentration of inhibitor solution. In the long term acidification experiments possible complications due to stomatal response (15) were removed by slicing leaves parallel to the epidermis, thus permitting free gas exchange. Changes in acidity were measured by shredding leaves into boiling deionized water and extracting for a further 20 min. On cooling, these extracts were immediately titrated to the bromocresol green end point with 0.1 N NaOH. Results are expressed as equivalents of acidity per unit fresh weight.

For the studies *in vitro* 5 g of leaves were homogenized in 20 ml of 50 mM tris-Cl (pH 7.5) containing 5 mM $MgCl_2$ and 5 mM 2-mercaptoethanol with 0.5 g of Polyclar-AT, filtered through Miracloth and centrifuged for 7 min at 20,000*g*. The supernatant obtained in this way did not interfere with the activity of several crystalline enzymes added as internal standards (7), and it was used as a source of malate dehydrogenase and P-enolpyruvate carboxylase.

Figure 1 shows the time course of the effect of 10 mM α-HPMS on $^{14}CO_2$ fixation in *A. hastata* and *A. spongiosa*. In both species, cut leaves in water showed a small decline in $^{14}CO_2$ fixation with time when illuminated for 30 min. Treatment of *A. hastata* with 10 mM α-HPMS had no effect on $^{14}CO_2$ fixation whereas, in *A. spongiosa*, $^{14}CO_2$ fixation was depressed by some 80% within 6 to 12 min of treatment. The rate of transpiration remained more or less steady throughout these experiments, suggesting that stomatal response (15) was not involved, and that inhibitor solutions were carried to mesophyll cells of both species. Other experiments showed that GBS was equally effective at 10 mM, but 1 mM solutions had little effect on $^{14}CO_2$ fixation. Sodium bisulfite (10 mM) was much less effective as an inhibitor of $^{14}CO_2$ fixation in *A. spongiosa* leaves.

The distribution of ^{14}C among products of 30-sec $^{14}CO_2$ fixation in *A. spongiosa* and *A. hastata* leaves is shown in Table I. These data were obtained from the final control and treatment samples shown in Figure 1 (25–32 min after treatment in water or 10 mM α-HPMS). α-HPMS appears to be an effective inhibitor of glycolate oxidase in both species, as shown by the accumulation of labeled glycolate and the inhibition of the labeling of glycine and serine. In addition, in *A. spongiosa* 10 mM α-HPMS vastly reduced the amount of label found in products of the initial carboxylation reaction (aspartate and malate) and in glycerate-3-P. In *A. hastata* leaves there was also evidence for the inhibition of the P-enolpyruvate carboxylase sequence (*cf.* References 2, 10) but no effect on the products of the Ru-1,5-dp carboxylase sequence. Similar results were found after treatment with GBS. These data suggest that the P-enolpyruvate carboxylase system is very sensitive to bisulfite compounds and that in *A. spongiosa* the bulk of the label in glycerate-3-P is derived from products of this reaction.

The dark acidification of succulent tissues is also mediated by a P-enolpyruvate carboxylase sequence (12, 13). *Sedum praealtum*

[1] On leave from Botany Department, University of Singapore, Singapore, 10.

[2] Abbreviations: α-HPMS: α-hydroxypyridine methane sulfonate; GBS: glyoxal bisulfite.

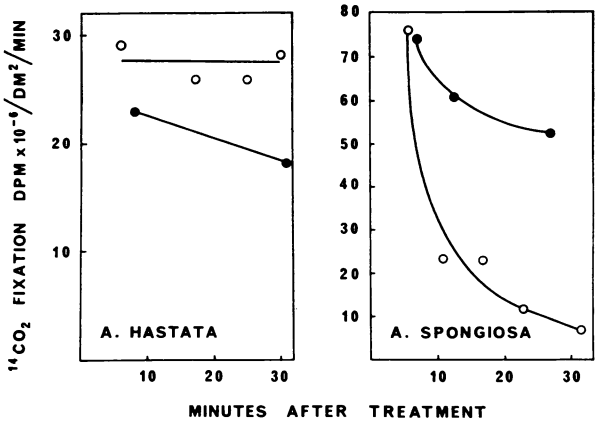

FIG. 1. Time course of the effect of bisulfite compounds on $^{14}CO_2$ fixation in two species of *Atriplex*. Leaves with petioles in water (●) and in 10 mM α-HPMS (○). Transpiration rates during the experiment were (in mg·dm^{-2}·min^{-1}), for *A. hastata*: control, 21 to 32; α-HPMS, 38 to 49; and for *A. spongiosa*: control, 23 to 28; α-HPMS, 59 to 64.

Table I. *Distribution of Radioactivity among Soluble Products after Photosynthesis in $^{14}CO_2$*

Photosynthesis was carried out for 30 sec, and leaves were treated in 10 mM α-HPMS or water for 25 to 32 min prior to exposure to $^{14}CO_2$. Percentage distribution in radioactivity applied to chromatograms shown in parentheses. Zero indicates below level of detection.

	A. spongiosa (C_4 type)		*A. hastata* (C_3 type)	
	Control	α-HPMS, 10 mM	Control	α-HPMS, 10 mM
	dpm 10^{-6}/dm^2			
Total ^{14}C fixed	26.2	3.5	9.2	14.1
Aspartate	16.20 (54.3)	0.45 (13.0)	1.33 (15.1)	0.15 (1.2)
Malate	5.08 (17.1)	0.42 (12.1)	0.34 (3.8)	0.22 (1.8)
Glycolate	0 (0)	0 (0)	0.23 (2.6)	0.77 (6.2)
Glycine-serine	0.02 (0.7)	0 (0)	0.73 (8.3)	0.33 (2.7)
Glycerate-3-P	4.83 (16.2)	0.16 (4.7)	3.93 (44.5)	6.54 (52.7)
Sugar phosphates	1.07 (3.6)	0.76 (21.9)	1.35 (15.3)	2.99 (23.3)
Neutral compounds	0.36 (1.2)	0.72 (20.8)	0.23 (2.6)	0.69 (5.6)

leaves used in these experiments showed at least 90% of label in malate after 30 min of dark $^{14}CO_2$ fixation, as found in other "Crassulacean" species (1). Acidification in the dark and deacidification in the light were both strongly inhibited in leaves injected with 10 mM α-HPMS (Fig. 2, a, b). Figure 3 shows that α-HPMS and GBS were very effective inhibitors of acidification and deacidification at concentrations of 5 to 10 mM. Sodium bisulfite itself was much less effective as an inhibitor of acidification and was without effect during deacidification (*cf.* $^{14}CO_2$ fixation in *A. spongiosa*).

Figure 4 shows the inhibition *in vitro* of P-enolpyruvate carboxylase and NADH malate dehydrogenase from *A. spongiosa* by α-HPMS and GBS. As indicated in the experiments *in vivo* (Fig. 2), GBS was the more effective inhibitor at the same concentration. Inhibition of P-enolpyruvate carboxylase appears to be competitive while that of malate dehydrogenase is of a mixed type. Identical results were obtained when P-enolpyruvate carboxylase was assayed directly by the incorporation of $^{14}CO_2$ into oxaloacetate. Similar results have been obtained with these en-

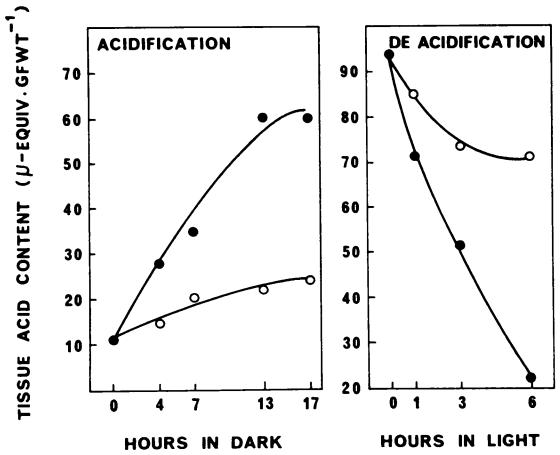

FIG. 2. Inhibition of dark acidification and deacidification in the light in *Sedum* leaves treated with 10 mM α-HPMS (○), compared with controls (●).

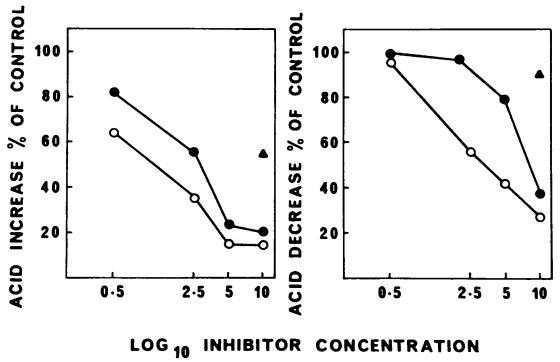

FIG. 3. Inhibition of dark acidification and deacidification in the light in *Sedum* leaves as a function of concentration of α-HPMS (●), GBS (○), and sodium bisulfite (▲).

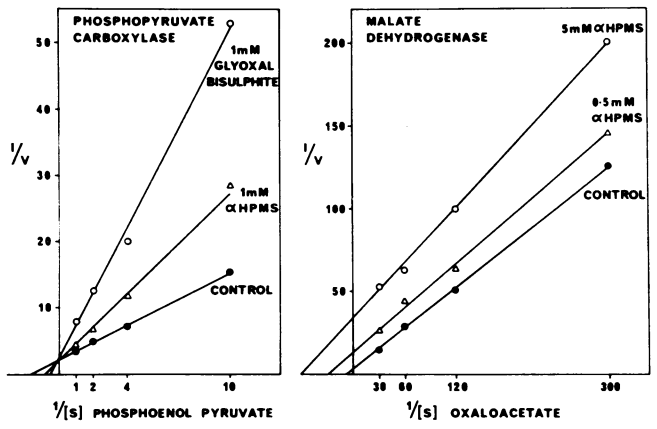

FIG. 4. Inhibition of P-enolpyruvate carboxylase and NADH malate dehydrogenase in *A. spongiosa* by α-HPMS and glyoxal bisulfite. P-enolpyruvate carboxylase assays contained, in 3 ml, 0.25 μmole of NADH; 5 units of crystalline malate dehydrogenase; 130 μmoles of tris-Cl, pH 7.4; and 20 μmoles of $MgCl_2$ with P-enolpyruvate, inhibitor, and extract at specified concentrations. The reaction was initiated by addition of substrate. In the presence of highest inhibitor concentrations malate dehydrogenase activity was more than adequate to couple this reaction. The malate dehydrogenase assays contained, in 3 ml, 0.25 μmole of NADH; 130 μmoles of tris-Cl, pH 7.4; extract; and the specified concentrations of oxaloacetate and inhibitor. The reaction was initiated by addition of substrate.

zymes prepared from maize, spinach, and *A. hastata* leaves, and with crystalline pig heart malate dehydrogenase. Thus far we have been unable to prepare extracts showing satisfactory activity of either enzyme from *Sedum* leaves by these methods.

Effects of bisulfite compounds on reactions other than glycolate oxidase have been indicated in other studies with green tissues (2, 6). Although the data presented here preclude the use of these compounds in relation to glycolate metabolism in C_4 plants during $^{14}CO_2$ fixation (7, 11), their effectiveness as inhibitors of P-enolpyruvate carboxylation may be a very useful tool. For example, it may now be possible to determine whether conventional C_3 type photosynthesis occurs at the same time as CO_2 fixation via phosphopyruvate carboxylase in C_4 plants or whether all CO_2 fixed passes through C_4 compounds in these species, as suggested here and in other experiments (3). The use of bisulfite compounds to inhibit Crassulacean acid metabolism under otherwise favorable conditions may permit detection of the suspected intermediates involved in the double carboxylation sequence proposed to account for the C_1/C_4 labeling pattern in malate (1). That bisulfite compounds inhibit the light deacidification process suggests that some of the CO_2 produced during decarboxylation of dark-synthesized malate in the light (4) may be converted to carbohydrate by C_4-type photosynthetic reactions, rather than the conventional C_3 pathway. These experiments will be discussed in detail elsewhere. CO_2 fixation and carbohydrate synthesis in species possessing the C_4 photosynthetic pathway and those showing Crassulacean acid metabolism suggest several analogies, and the comparative biochemistry of these processes is being studied.

The data presented show bisulfite compounds to be inhibitors of the P-enolpyruvate carboxylase system, in addition to glycolate oxidase (14). Of particular interest is the stomatal response to these compounds (16, 6). The role of CO_2 and of carboxylation reactions in stomatal opening has recently been reemphasized (5), and it may be that bisulfite compounds act directly on these reactions.

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