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GLYCOLLATE OXIDASE INHIBITION AND ITS EFFECT ON PHOTOSYNTHESIS AND PIGMENT FORMATION IN *ZEA MAYS*

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Key Word Index—*Zea mays*; Gramineae; maize; glycollate oxidase; photorespiration; photosynthesis; 2-hydroxy-3-butynoic acid; pigment formation; greening.

Abstract—We have investigated the effect of 2-hydroxy-3-butynoic acid (HBA) and its methyl ester (MeHBA) on photosynthesis and pigment formation in *Zea mays*, a C_4 photosynthesis-type plant. In the presence of the specific inhibitor of glycollate oxidase, assimilation of CO_2 was decreased significantly. Labelling patterns showed accumulation of glycollate, though not so marked as in C_3 photosynthesis-type plants, and marked decreases in incorporation into glycine, serine and particularly glycerate. This inhibition was specific for the *S*(+) enantiomers of HBA and MeHBA. In greening maize *R,S*-MeHBA inhibited formation of chloroplast pigments and this effect could be shown to be due to the S(+) enantiomer; of a range of metabolites tested only supplementations with serine or pyruvate were partly effective in restoring greening.

INTRODUCTION

We have previously reported[1] the effect of 2-hydroxy-3-butynoic acid (HBA) and its methyl ester (MeHBA) on photosynthesis in barley (*Hordeum vulgare*). HBA has also been used to inhibit photorespiration in other C_3 plant types, including *Triticum aestivum*[2], *Glycine max*[3, 4] *and Helianthus annuus*[5]. These inhibitors are potent and irreversible inhibitors of glycollate oxidase[6] and caused greatly decreased assimilation of CO_2, while labelling patterns showed a massive accumulation of glycollate; effects were specific to the *S*(+) enantiomers of HBA and MeHBA [1]. In greening barley the *S*(+) enantiomers inhibited formation of chloroplast pigments in parallel with inhibition of glycollate oxidase. This was the only enzyme of the glycollate pathway whose activity was significantly decreased after inhibitor treatments.

Most studies with inhibitors of glycollate metabolism have been carried out with plants of the C_3 photosynthesis type, since these represent the greatest potential for improvement in crop yield if photorespiration can be decreased. Work on glycollate metabolism in C_4 plants has been fragmentary, though studies with *Zea mays* using α-hydroxypyridinemethanesulphonic acid have been reported[7] and butyl hydroxybutynoate has been used as an inhibitor with *Panicum miliaceum*[8]. In their comparative study these latter workers also included *Hordeum vulgare* as an example of a C_3 photosynthesis type. We now report investigations into the effect of the *S*(+) and *R*(−) enantiomers of HBA and MeHBA on the path of photosynthetically fixed carbon in *Zea mays*, and on the consequence of glycollate oxidase inhibition by these hydroxybutynoate compounds on greening of etiolated maize seedlings.

RESULTS AND DISCUSSION

Effects on photosynthetic CO_2 *assimilation*

As for previous studies with *Hordeum vulgare* an equilibration period of at least 60 min illumination in the leaf chamber of the assimilation apparatus was allowed before exposure to $^{14}CO_2$ to ensure that steady-state photosynthesis was attained in the leaf sections. Under these conditions the net assimilation rate for untreated maize, calculated from the specific radioactivity of $^{14}CO_2$ in the feed gas, was 12.6 mg $CO_2/dm^2/hr$. This rate compares favourably with that of 11.3 mg $CO_2/dm^2/hr$ obtained for *Zea mays* by other workers[9] but is considerably less than the values of *ca* 60 mg $CO_2/dm^2/hr$ which can be obtained for C_4 plants under optimum natural conditions[10].

Net ^{14}C-assimilation into water-soluble compounds for leaf tissue exposed to *S*(+) and *R*(−) enantiomers of HBA and MeHBA, compared with that of the untreated control, is shown in Table 1. ^{14}C-incorporation was decreased *ca* 46% by 0.5 mM *S*(+)HBA and 44% by 1.0 mM *S*(+)MeHBA. In contrast, the *R*(−) enantiomers were much less effective, and the effect of 1.0 mM *R*(−)MeHBA in decreasing CO_2 assimilation by 11 and 15% in two experiments can be attributed to partial racemization to the *S*(+) enantiomer. In the case of *R*(−)HBA and a lower concentration of *R*(−)MeHBA there were slight increases in CO_2 assimilation compared with that of the untreated control.

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Table 1. Effect of HBA and MeHBA enantiomers on net ^{14}C-assimilation into water-soluble material of maize leaf sections following 30 min photosynthesis in $^{14}CO_2$–air

Pre-treatment	^{14}C incorporated ($10^{-6}\times$ dpm)		% reduction or increase	
No inhibitor	54.5	64.4	—	—
S(+)MeHBA 0.2 mM	52.5	—	−3.7	—
1.0 mM	40.5	—	−25.7	—
1.0 mM	—	35.9	—	−44.2
R(−)MeHBA 0.2mM	63.7	—	+14.4	—
1.0 mM	46.1	—	−15.4	—
1.0 mM		57.1	—	−11.3
S(+)HBA 0.5 mM	—	35.0	—	−45.7
R(−)HBA 0.5 mM	—	68.6	—	+6.5

Two primary leaf sections of maize (total area *ca* 10 cm^2) from 2-week-old seedlings were arranged in frames, with the cut bases in water or solution as indicated, and illuminated (*ca* 5 klx, 85 μmol/m^2/sec; temp. *ca* 25°) and flushed with normal air for 60 min, then with $^{14}CO_2$–air containing 25 μ Ci^{14}C/l. for 30 min (both at 0.8 l/min) before rapid killing, extraction and estimation of ^{14}C in water-soluble material. Data given are from two representative experiments.

In each case the leaf material was extracted and the ^{14}C-labelling pattern amongst metabolites following photosynthetic $^{14}CO_2$ assimilation was assessed (Table 2). The data presented for the untreated leaf tissue were typical of several experiments as judged by ^{14}C distribution following autoradiography. Though resolution into some 20 radioactive zones was achieved, only the 12 zones representing compounds of most interest, and accounting for *ca* 90% of the total incorporation, were assayed for ^{14}C.

It is widely accepted that plants with the (additional) C_4 pathway of carbon assimilation generally have higher rates of photosynthesis than C_3 plants (see e.g. refs. [10–12]). C_4 photosynthesis is associated with small rates of photorespiratory CO_2 release in the light (and hence low CO_2-compensation concen-

Table 2. Effects of *S*(+)HBA, and *S*(+)MeHBA enantiomers on ^{14}C-assimilation into water-soluble products of maize leaf sections following photosynthesis in $^{14}CO_2$–air

Treatment	^{14}C incorporated ($10^{-5}\times$ dpm)			
	No inhibitor	*S*(+)HBA 0.5 mM	*S*(+)MeHBA 1.0 mM	*R*(−)MeHBA 1.0 mM
Total	643.6 (100%)	350.5 (100%)	358.5 (100%)	570.7 (100%)
Compounds				
PGA	26.8 (4.2)	8.8 (2.5)	8.6 (2.4)	26.5 (4.6)
HMP	45.3 (7.0)	31.4 (9.0)	25.5 (7.1)	37.8 (6.6)
HDP	1.7 (0.3)	0.9 (0.3)	2.0 (0.6)	1.8 (0.3)
Sucrose	341.0 (53.0)	141.8 (40.5)	151.7 (42.3)	334.9 (58.7)
Glycollate	Neg. (<0.1)	49.4 (14.1)	28.2 (7.9)	1.5 (0.3)
Glycine	6.6 (1.0)	Neg. (<0.1)	3.0 (0.8)	7.6 (1.3)
Serine	10.3 (1.6)	4.2 (1.2)	6.0 (1.7)	14.7 (2.6)
Glycerate	6.5 (1.0)	Neg. (<0.1)	Neg. (<0.1)	6.5 (1.1)
Alanine	16.3 (2.5)	17.2 (4.9)	3.6 (1.0)	14.0 (2.5)
Malate	89.4 (13.9)	39.1 (11.2)	59.4 (16.6)	73.9 (13.0)
Aspartate	30.1 (4.7)	28.8 (8.2)	17.0 (4.7)	35.0 (6.1)

Neg., negligible.

Experimental details were as for Table 1. The ^{14}C-distribution pattern for *R*(−)HBA was similar to that for *R*(−)MeHBA. PGA, phosphoglycerate; HMP, hexose monophosphates; HDP, hexose diphosphates.

trations) which has been attributed to much smaller rates of carbon flux in the glycollate pathway compared with that in C_3 plants. Comparison of the ^{14}C-assimilation pattern for maize following long-term photosynthesis in $^{14}CO_2$–air, when compared with that for the C_3 plant type, *Hordeum vulgare*, previously studied by the same methods[1] is consistent with these views. Incorporation into the photorespiratory intermediates glycollate, glycine and serine in maize was substantially less than in barley, and formed a smaller percentage of the total. These data agree with the accepted view that the oxygenation reaction catalysed by ribulose bisphosphate carboxylase leading to phosphoglycollate formation, and hence photorespiration, occurs to a lesser extent in C_4 species compared with C_3 species owing to greater concentration of CO_2 at the site of the enzyme, the bundle sheath cells, in C_4 plants[11].

After prolonged photosynthesis in $^{14}CO_2$–air, the levels of radioactivity in the early products of photosynthesis represent the relative pool sizes of these intermediates. The greater photosynthetic efficiency of maize compared with earlier results for barley[1] is reflected in greater ^{14}C-incorporation into the Calvin cycle intermediates PGA, HMP and HDP, and into sucrose. Appreciable incorporation into malate was also observed consistent with this C_4 acid being responsible for the bulk of transfer of assimilated carbon from the mesophyll to the bundle sheath cells (e.g. refs.[10, 11]). [^{14}C]Alanine, which may be formed via transamination from the C_4 pathway intermediate pyruvate, was also accumulated to a somewhat greater extent than in barley.

Following treatment of maize leaf sections with *S*(+)HBA or *S*(+)MeHBA an accumulation of ^{14}C in glycollate was observed. The relative importance of glycollate in maize compared with that in the C_3-photosynthetic type plant is emphasized by the observation that glycollate in HBA or MeHBA treated maize tissue accounted for up to 15% of the total ^{14}C assimilated, whereas in experiments with barley it accounted for 50–80%[1]. In other respects the effects of inhibition were similar. In maize marked decreases in incorporations into glycine, serine and particularly glycerate were seen. Incorporation into malate and other compounds paralleled the decrease in total $^{14}CO_2$ assimilated, though incorporations into alanine and aspartate appeared to be decreased more following *S*(+)MeHBA treatment than with *S*(+)HBA. At 0.2 mM *S*(+) MeHBA (data not shown) similar effects were less marked, though here ^{14}C-incorporations into alanine and aspartate were apparently enhanced, and that into malate decreased. In contrast to the *S*(+) enantiomers, *R*(−) HBA and *R*(−)MeHBA were ineffective and ^{14}C-distribution patterns remained very similar to those of the untreated control.

In *Hordeum vulgare*[1] HBA and MeHBA inhibited glycollate oxidase activity by *ca* 90%. Assay of other enzymes of the glycollate pathway confirmed that glycollate oxidase was the site of action of the inhibitor. Comparable studies with *Zea mays*, where normal amounts of the enzyme were *ca* 10% of those in the C_3 plant, showed (Fig. 1) that glycollate oxidase was inhibited some 80% within 2 hr of exposure to MeHBA.

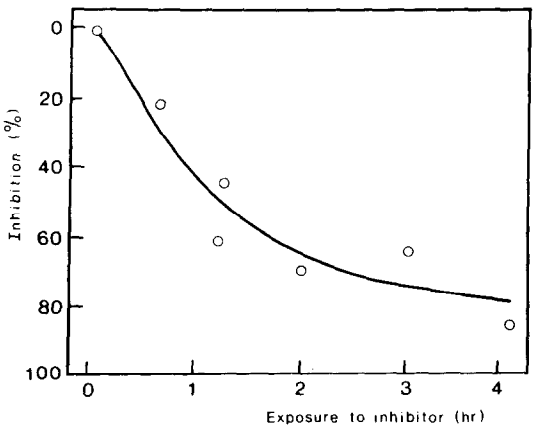

Fig. 1. Inhibition of glycollate oxidase in etiolated maize leaf segments by MeHBA. Five leaf sections (*ca* 4 cm long) of etiolated 8-day-old maize were stood with cut bases in 1.5 mM *R,S*-MeHBA. Samples were incubated in darkness for the time indicated before glycollate oxidase estimation. Enzyme activity in the untreated control was 0.09 μ mol/min/g fr. wt.

Effect on pigment formation

The availability of a specific inhibitor of glycollate oxidase made possible experiments to test the contribution of the glycollate pathway to chloroplast pigment formation in greening tissue. Such a role has been suggested in chlorophyll formation[13] and in the synthesis of chloroplast terpenoids[14].

As in previous studies[15], experiments were carried out using etiolated leaf segments floated on 2.5 mM potassium phosphate buffer (pH 7), containing additions as necessary. In contrast to barley[1] pigment accumulation was markedly decreased at higher pH's and at pH's below 6.0 and consequently experiments where the pH at the conclusion of incubations was outside the range 6.0–7.5 were discarded. Over a 30 hr period at 4 klx illumination, untreated tissue typically formed *ca* 700 μg chlorophyll/g fr. wt and gave an A_{480}^{car} of 40/g fr. wt. Chlorophyll concentrations after this time were *ca* 60% those in normal green leaf of the same age. The optimal age of maize seedlings for such greening experiments was 2–3 days after leaf unrolling, *ca* 8 days from germination.

The effect of HBA and MeHBA on the greening of etiolated maize was followed by exposing leaf segments to various concentrations of MeHBA (Fig. 2). Chlorophyll formation was inhibited *ca* 75% by 1.5 mM *R,S*-MeHBA. In this experiment the effect on carotenoid formation was somewhat less (*ca* 55%) but this was more difficult to assess because of the appreciable amount of carotenoid present in etiolated maize. As expected, higher concentrations of MeHBA decreased chlorophyll formation even further, but carotenoid formation appeared to be maximally inhibited at 0.8 mM inhibitor. The relative effectiveness of the *S*(+) and *R*(−) enantiomers of HBA and MeHBA was also tested (Table 3). Chlorophyll formation was inhibited *ca* 60% by the *S*(+) enantiomer of both inhibitors at 0.75 mM, and comparable inhibition was obtained by twice this concentration of the racemic

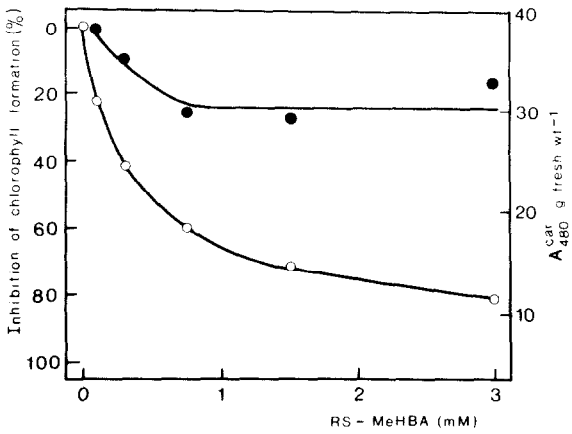

Fig. 2. Inhibition of pigment accumulation in illuminated etiolated maize leaf segments by *R,S*-MeHBA. In incubations *ca* 0.5 g of 8-day-old maize leaf segments, *ca* 1 cm long, were floated on 20 ml water or *R,S*-MeHBA solution at the concentration indicated. Samples, in lidded Petri dishes, were incubated for 3 hr in darkness then illuminated (*ca* 4 klx) for 48 hr at 26° before chlorophyll (○) and carotenoid (●) estimation. The levels of chlorophyll accumulated in control samples was 420 μg/g fr. wt; etiolated carotenoid value was 21.1 A^{car}_{480}/g fr. wt.

mixture of MeHBA. In contrast, the *R*(−) enantiomers were much less effective, the partial inhibitions shown being attributable to some racemization to the *S*(+) form, this being more significant for HBA than for its methyl ester [Jewess, P. J., personal communication]. Here inhibition of carotenoid formation was similar to that observed for chlorophyll, with a more marked distinction between the effects of the *S*(+) and *R*(−) enantiomers.

The conviction based on previous studies[1, 2] that the site of action of HBA in greening maize was glycollate oxidase was confirmed by experiments in which etiolated maize segments were exposed via their cut bases to 1.5 mM MeHBA (Fig. 1). Glycollate oxidase activity was inhibited by almost 75% within 2 hr of exposure to inhibitor. As with barley ([1] but cf. ref.[8]) glycollate oxidase was not totally inhibited even by prolonged exposure to inhibitor. This may be because the glycollate which accumulates as a result of the inhibition partially protects the FMN cofactor of glycollate oxidase from interaction with HBA.

Attempts to restore greening following MeHBA treatment by supplementation of the incubation medium with various metabolites implicated in glycollate metabolism proved ineffective, though marginal improvements with serine and pyruvate were seen in some experiments (maximally 50 and 65% greening, compared with *ca* 35% greening for tissue in the presence of 0.75 mM MeHBA). Suggested reasons for the inability to restore greening in barley treated with MeHBA, other than by a few metabolite combinations, have been given previously[1]. Nevertheless, the present experiments convincingly demonstrate that specific inhibition of glycollate oxidase in greening tissue by the stereospecific *S*(+) enantiomer of HBA is accompanied by a parallel inhibition of chloroplast pigment formation.

EXPERIMENTAL

Growth of seedlings. Seeds of *Zea mays*, variety Dekalb-202, were obtained from Miln Masters Group Ltd., Chester, U.K., and grown in Vermiculite or soil in an environmental

Table 3. Inhibition of pigment accumulation in illuminated etiolated maize leaf segments by HBA and MeHBA enantiomers

		Pigment accumulated		
		Carotenoid	Chlorophyll	
		A^{car}_{480}/g fr. wt	μg/g fr. wt	% greening
Controls*				
	E.T.	18.5	—	—
	D.	24.7	38†	0
	G.T.	38.0	717†	100
Inhibitor	(mM)			
R(−)HBA	0.75	34.9	475	64
S(+)HBA	0.75	29.6	315	41
R(−)MeHBA	0.75	37.9	555	76
S(+)MeHBA	0.75	29.0	300	39
R,S − MeHBA	1.5	31.2	290	37

*E.T., etiolated tissue; D., dark control, tissue incubated in darkness; G.T., greened tissue, incubated in light.

†These values form the basis for calculation of % greening in each experiment.

1 g of etiolated 8-day-old maize leaf segments, *ca* 1 cm long, were floated on 20 ml 2.5 mM KPi buffer (pH 7), containing inhibitor as indicated. Samples, in lidded Petri dishes, were incubated in darkness for 3 hr then illuminated (*ca* 4 klx; 65 μ mol/m^2/sec) for 48 hr at 26° before pigment estimation.

growth chamber. Etiolated seedlings were grown in darkness at 25°, while for normal (green) seedlings growth was under a 14-hr regime (day temp. 26°, night temp. 20°) with fluorescent and tungsten lights providing 8 klx at the leaf surface (130 μmol/m^2/sec).

Other methods. Greening studies and studies on $^{14}CO_2$ assimilation were as described in ref.[1]. Leaf material used in the leaf chambers of the assimilation apparatus had a total area of *ca* 10 cm^2 (2 leaf sections; *ca* 250 mg fr. wt.)

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