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GLYCOLLATE OXIDASE INHIBITION AND ITS EFFECT ON PHOTOSYNTHESIS AND PIGMENT FORMATION IN *HORDEUM VULGARE*

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

Abstract—Investigations of the effect of 2-hydroxy-3-butynoic acid and its methyl ester on photosynthesis in *Hordeum vulgare* are reported. In the presence of either of these compounds the assimilation of $^{14}CO_2$ was greatly decreased. The labelling patterns showed massive accumulation of glycollate and greatly reduced incorporation into sucrose and other products of photosynthesis. The inhibition was specific for the *S*(+) enantiomers. In greening barely the *S*(+) enantiomers inhibited formation of chloroplast pigments, and this was paralleled by inhibition of glycollate oxidase. This was the only enzyme of the glycollate pathway whose activity was significantly decreased after inhibitor treatments. Of a range of metabolites tested, only supplementations with glycine and glutamate or glycine, serine and succinate fully restored greening.

INTRODUCTION

Following the demonstration [1] that lactate oxidase from *Mycobacterium smegmatum* can be inhibited by the acetylenic substrate analogue 2-hydroxy-3-butynoic acid (HBA), it was shown [2] that both HBA and its methyl ester (MeHBA) would inhibit glycollate oxidase (glycollate: oxygen oxidoreductase, EC 1.1.3.1) in higher plants within a few hours of uptake. MeHBA is thought to be activated *in vivo* by esterase-catalysed hydrolysis to HBA, and the latter proved to be an effective *in vitro* inhibitor of glycollate oxidase.

By comparison with the inhibition of other flavin-linked oxidases by acetylenic substrate analogues [3–5] the mechanism of inhibition (Fig. 1) is thought to involve reaction at position 4a of the isoalloxazine ring of FMN in an analogous manner to the natural substrate, but resulting here in the formation of a modified cofactor, incapable of further redox reactions. The inhibitor therefore competes with glycollate for the active site of the enzyme, but its effect is irreversible. Clearly the inhibitor would be expected to show high specificity, and since glycollate oxidase is stereospecific towards homologous α-hydroxyacids higher than glycollate only the *S*(+) enantiomer should inhibit the enzyme. This is the case for glycollate oxidase isolated from *Pisum sativum* and treated *in vitro* with the HBA enantiomers [Jewess, P. J., personal communication].

In higher plants the general view is that glycollate is formed in the chloroplast, via phosphoglycollate, through the oxygenase activity of ribulose bisphosphate carboxylase–oxygenase. The product of its oxidation, glyoxylate, is further metabolized in photorespiration by the pathway outlined in ref. [6], ultimately returning to the chloroplast as 3-phosphoglycerate. This pathway, which involves co-operation of peroxisomes and mitochondria, is particularly active in plants with C_3 photosynthesis. HBA has therefore proved useful in studies of the effect of inhibition of the glycollate pathway on carbon flow in photosynthesis and related pathways in *Triticum aestivum* [7], *Glycine max* [8, 9] and *Hordeum vulgare* [10]. In this paper, we report investigations into the effect of the *S*(+) and *R*(−) enantiomers of HBA and MeHBA on the path of photosynthetically fixed carbon in *H. vulgare*.

Although some workers consider the glycollate pathway to be an unnecessary and wasteful process (see e.g. ref. [11]) others have suggested possible roles. For example, the pathway may be partly responsible in C_3 plants for the transport of fixed carbon and energy from the chloroplast to support sucrose synthesis in the cytoplasm [12, 13]. In contrast to earlier views [14–16] there is appreciable evidence that chloroplasts may have the potential to synthesize chloroplast terpenoids [17] and acyl residues of acyl lipids [18] from CO_2 via pyruvate dehydrogenase [19, 20]. In etiolated tissues undergoing greening, possible roles for glycollate in the formation of chlorophyll [21–23] and chloroplast terpenoids [24] have been suggested. We have therefore also looked at the effect of glycollate oxidase inhibition on greening of barley.

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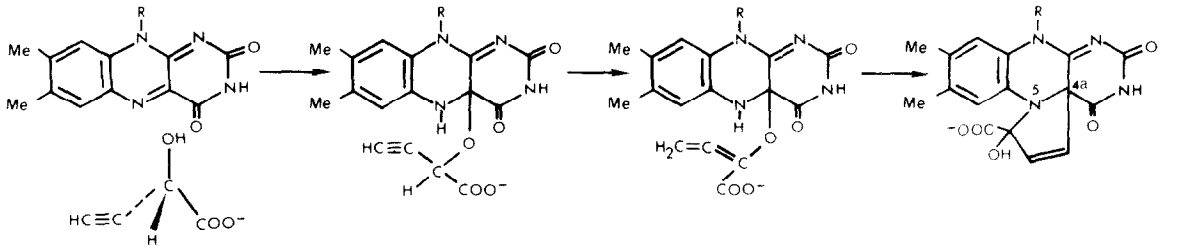

Fig. 1. Suggested reaction mechanism for FMN in glycollate oxidase with the acetylenic substrate analogue *S*(+) HBA (based on ref. [5]). R = D-Ribitol phosphate.

RESULTS AND DISCUSSION

Effect on photosynthetic CO_2 *assimilation*

Before treatments with the inhibitors were carried out it was confirmed that steady-state photosynthesis was attained in leaf sections under the experimental conditions used. Assimilation of $^{14}CO_2$ into extractable ethanol–water material during a 6 min exposure to $^{14}CO_2$–air showed that a constant rate of incorporation was attained within 30 min of commencing illumination and was sustained for several hours. An equilibration period of 60 or 120 min illumination before exposure to $^{14}CO_2$ was used in subsequent experiments.

During a 30 min exposure to $^{14}CO_2$, assimilation of ^{14}C was linear with time, and incorporation into leaf sections exposed 24 hr earlier to 1.0 mM MeHBA for 4 hr was only *ca* 10% that of untreated controls. For untreated leaf sections (Fig. 2a), ^{14}C incorporation into neutral compounds, mainly sugars, increased with time and these became the predominantly labelled fraction. The lipid fraction showed a similar trend but incorporation of ^{14}C into these components was substantially lower. A basic fraction containing amino acids contained the greatest amount of ^{14}C at the shortest exposure times, but this then progressively decreased as a percentage of the ^{14}C assimilated as ^{14}C flux through these components approached the steady state. At very short exposure times the acidic fraction, containing the phosphate esters of the photosynthetic carbon reduction cycle, should be labelled first. However, by 2.5 min, the shortest analysis time in this experiment, the incorporation into this fraction was only some 20% of the total ^{14}C assimilated, though the increased assimilation into this fraction with time meant this percentage remained constant over the remainder of the experiment.

In leaf sections from seedlings which had previously taken up MeHBA via the roots (Fig. 2b) ^{14}C incorporation was considerably lower. Here incorporations into lipid and basic fractions showed similar trends to those seen in untreated controls. However, in contrast to the data for untreated controls the acidic fraction became predominantly labelled and substantially less $^{14}CO_2$ was incorporated into the neutral fraction. At steady-state incorporations attained after 10 min exposure to $^{14}CO_2$, these fractions contributed *ca* 55 and 15%, respectively, of total ^{14}C incorporated. These data indicate that partial inhibition of glycollate oxidase results in the accumulation of acid components, presumably glycollate, at the expense of sugars. That complete inhibition of the enzyme had not occurred is suggested by similar kinetics of labelling of the basic fraction

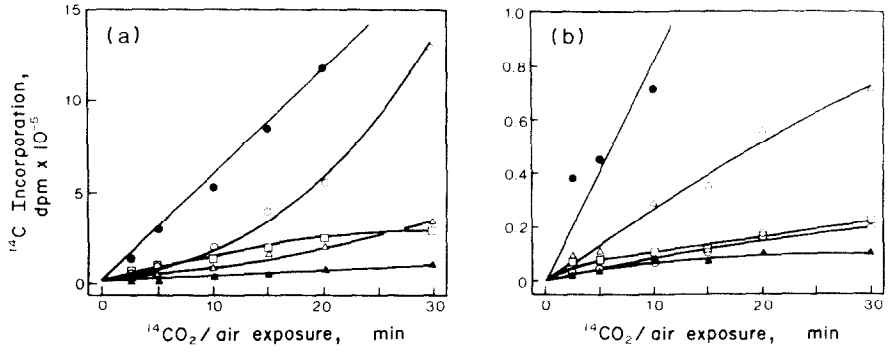

Fig. 2. ^{14}C Incorporation following photosynthesis in $^{14}CO_2$–air into various extractable fractions of barley leaf sections from (a) untreated seedlings, and (b) seedlings previously treated with MeHBA via the roots. 2-week-old barley seedlings were stood with their roots immersed in nutrient solution alone or containing 1.0 mM MeHBA for 4 hr before transfer to solution not containing inhibitor. After a further 24 hr under normal growth conditions leaf sections were illuminated (*ca* 25 klx) and flushed with normal air for 60 min, then with $^{14}CO_2$–air containing 1 μCi ^{14}C/l. for the time indicated before rapid extraction, and estimation of ^{14}C in various fractions. (○), Neutral fraction (mainly sugars); (△), acidic fraction (mainly organic acids and phosphate esters); (□), basic fraction (mainly amino acids); (▲), lipid fraction; (●), total incorporation.

and that, although it was markedly decreased, some incorporation of ^{14}C into the neutral fraction still occurred. That this labelling comes from alternative paths not involving glycollate metabolism cannot, however, be discounted. It was confirmed that in leaf sections from plants exposed to 1.0 mM MeHBA for several hours glycollate oxidase was some 80% inhibited (Fig. 3).

H. vulgare was included in a comparative survey [10] of the effect of butyl hydroxybutynoate on plants of C_3, C_4 and intermediate photosynthetic type. Though direct comparison is difficult since these workers adopted the somewhat different approach of studying incorporation in different fractions after various times of exposure to inhibitor, our results are in general agreement. Results are also consistent with those reported for the effect of HBA on *Triticum vulgare* [7].

The ^{14}C labelling pattern amongst metabolites following photosynthetic $^{14}CO_2$ fixation in the presence of inhibitor was also studied. Experiments with *S*(+) and *R*(−) enantiomers of MeHBA and HBA were carried out, leaf segments being exposed to inhibitor via the cut bases while in the leaf chamber of the assimilation apparatus. Under typical steady-state photosynthesis conditions, the net assimilation rate for untreated controls in these experiments, calculated from the specific radioactivity of $^{14}CO_2$ in the feed gas, was 6.7 mg $CO_2/dm^2/hr$. Although relatively low by comparison with intact plants under optimum natural conditions, where values as high as 20 mg $CO_2/dm^2/hr$ can be obtained (see e.g. ref. [25]), these values compare favourably with the values of 2.6–5.9 mg $CO_2/dm^2/hr$ for wheat leaf sections in similar experiments at atmospheric levels of CO_2 and oxygen [7, 26]. Net ^{14}C assimilation into water-soluble compounds was decreased 72% by 1.0 mM *S*(+) MeHBA (Table 1); inhibitor at 0.2 mM proved less effective but some inhibition still occurred. *S*(+) HBA also proved markedly inhibitory. In contrast, the *R*(−) enantiomers were less potent; although at 1.0 mM the *R*(−) MeHBA enantiomer gave a 37% inhibition of

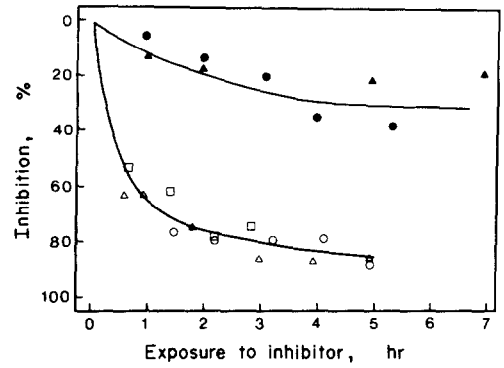

Fig. 3. Inhibition of glycollate oxidase in barley by MeHBA. Experiments were with leaf segments of etiolated plants or normal green seedlings. Leaf segments (*ca* 1 g) of etiolated 7-day-old seedlings were floated on a solution of MeHBA, without illumination, for the time indicated before glycollate oxidase estimation. Green barley seedlings, *ca* 2-week-old, were exposed via the roots to MeHBA for the time indicated, then were maintained under normal growth conditions (no inhibitor) for 24 hr before estimation of glycollate oxidase. Enzyme activities in untreated controls were *ca* 1.0 and 1.7 μmol/min/g fr. wt, respectively. (□), Leaf segments exposed to 1.0 mM MeHBA; (○), (△), seedlings exposed to 1.0 mM MeHBA; (●), (▲), seedlings exposed to 0.1 mM MeHBA.

^{14}C assimilation this was probably due to partial racemization to the *S*(+) enantiomer. In the case of *R*(−) HBA and the lower concentration of *R*(−) MeHBA, however, there was an apparent increase in ^{14}C assimilated.

Effects on radioactivity in intermediates

The ^{14}C distribution patterns obtained in these experiments are given in Table 2. The results for untreated barley were typical of several experiments as judged by ^{14}C distribution visualized by radioautography following TLC. In these studies resolution

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

Pre-treatment		Time (min)	^{14}C incorporated (10^{-6} × dpm)		% reduction (−) or increase (+)
No inhibitor		60	30.0	34.8	—
S(+) MeHBA	0.2 mM	60	28.2	—	−6.0
	1.0 mM	120	8.4	—	−72.0
R(−) MeHBA	0.2 mM	60	32.4	—	+8.0
	1.0 mM	120	18.8	—	−37.3
S(+) HBA	0.5 mM	60	—	24.7	−29.0
R(−) HBA	0.5 mM	60	—	38.9	+11.8

Four primary-leaf sections of barley (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; temp. *ca* 25°) and flushed with normal air for the time indicated, then with $^{14}CO_2$–air containing 25 μCi ^{14}C/l. for 30 min (both at 0.8 l/min) before extraction and estimation of ^{14}C in water-soluble material.

Table 2. Effects of HBA and MeHBA enantiomers on ^{14}C assimilation into water-soluble products of barley leaf sections following 30 min photosynthesis in $^{14}CO_2$–air

	^{14}C incorporated ($10^{-5} \times$ dpm)*					
Compounds isolated	No inhibitor	0.5 mM *S*(+) HBA	0.5 mM *R*(−) HBA	No inhibitor	1.0 mM *S*(+) MeHBA	1.0 mM *R*(−) MeHBA
Total	347.7 (100)	247.4 (100)	388.7 (100)	300.0(100)†	83.8 (100)	188.5 (100)
PGA	10.5 (3.0)	2.5 (1.0)	9.5 (2.5)	6.6 (2.2)	0.4 (0.5)	1.3 (0.7)
HMP	9.1 (2.6)	7.5 (3.0)	9.9 (2.6)	9.3 (3.1)	3.0 (3.6)	5.4 (2.9)
HDP	0.9 (0.3)	0.5 (0.2)	0.6 (0.1)	0.6 (0.2)	n.d.	0.8 (0.4)
Sucrose	185.8 (53.4)	59.7 (24.1)	210.9 (54.2)	134.7 (45.0)	4.1 (4.8)	46.9 (24.9)
Phosphoglycollate	0.6 (0.2)	Neg. (<0.1)	Neg. (<0.1)	0.1 (<0.1)	Neg. (<0.1)	Neg. (<0.1)
Glycollate	1.6 (0.4)	125.4 (50.7)	3.7 (0.9)	1.2 (0.4)	65.3 (78.0)	67.3 (35.7)
Glycine	28.4 (8.2)	3.7 (1.5)	31.8 (8.2)	26.8 (8.9)	1.3 (1.6)	3.9 (2.1)
Serine	29.0 (8.3)	4.5 (1.8)	38.0 (9.8)	20.2 (6.8)	0.3 (0.4)	14.8 (7.9)
Glycerate	4.3 (1.2)	0.6 (0.2)	4.4 (1.1)	5.7 (1.9)	1.3 (1.6)	1.3 (0.7)
Alanine	3.0 (0.9)	1.7 (0.7)	3.8 (1.0)	4.2 (1.4)	Neg. (<0.1)	Neg. (<0.1)
Malate	3.9 (1.1)	0.5 (0.2)	4.4 (1.1)	9.7 (3.3)	1.4 (1.6)	1.7 (0.9)
Aspartate	6.4 (1.8)	1.0 (0.4)	11.8 (3.0)	15.8 (5.2)	0.7 (0.8)	2.4 (1.3)

Experimental details were as for Table 1. Neg., negligible amounts; n.d., not determined.

*% total activity in water-soluble products given in parenthesis.

†Incorporations are the means of two experiments; in both cases total ^{14}C incorporation was the same.

into more than 20 radioactive zones was achieved though only the 12 zones representing the compounds of most interest in the present study were assayed for ^{14}C, these accounted for *ca* 85% of the total incorporation. Greatest variability in ^{14}C assimilation in these experiments was in glycine and serine, though the total of these was very similar, and in malate and aspartate.

Following treatment with *S*(+) HBA there is a massive increase in incorporation into glycollate (Table 2), which now accounts for 50% of the ^{14}C fixed. Amounts of phosphoglycollate were negligible following all treatments with *S*(+) HBA or *S*(+) MeHBA, suggesting that *in vivo* phosphoglycollate phosphatase is not subject to product inhibition.

For the earliest products of photosynthesis, such as phosphoglyceric acid (PGA), the long-term incorporation may reflect the steady-state pool size under photosynthesizing conditions. Radioactivity in PGA was hence routinely estimated as an indication of functioning of the Calvin cycle. Insufficient incorporation into, and less satisfactory resolution of, other phosphate ester intermediates of the Calvin cycle prevented accurate assessment, but hexose monophosphate (HMP) and diphosphate (HDP) zones were estimated as a further indication of the functioning of the Calvin cycle. These compounds were of interest because of their involvement in starch synthesis in the chloroplast. After treatment with *S*(+) HBA, incorporation into PGA was decreased, both quantitatively and as a percentage of the total. Incorporation into sucrose was also severely affected, suggesting a lack of suitable triose phosphates for its synthesis in the cytoplasm, though the amounts of HMP and HDP themselves were not markedly affected.

The incorporation of ^{14}C into alanine, and particularly malate and aspartate, was significantly decreased after *S*(+) HBA treatment. Radioactively labelled alanine and aspartate are derived via transamination of [^{14}C]pyruvate and [^{14}C]oxaloacetate, respectively [27]. Pyruvate becomes rapidly labelled during photoassimilation of $^{14}CO_2$ as a result of glycolytic metabolism of ^{14}C-labelled triose phosphate, exported from the chloroplast. Incorporation in oxaloacetate arises either from pyruvate, owing to tricarboxylic acid cycle activity, or from triose phosphate via PGA and phosphoenolpyruvate (PEP), the last step then catalysed by PEP-carboxylase incorporating further $^{14}CO_2$. ^{14}C-labelled malate can be derived from [^{14}C]oxaloacetate by the action of malate dehydrogenase. Since malate, oxaloacetate and aspartate may be involved in shuttles to export reducing power from the chloroplast [28] a rapid transfer of ^{14}C among these metabolites is possible. The decreased incorporation into such compounds after *S*(+) HBA treatment is therefore again consistent with a reduction in photosynthesis and supply of available triose phosphate. With *R*(−) HBA no marked differences from the control in distribution of ^{14}C among metabolites were observed.

Administration of 1.0 mM *S*(+) MeHBA to the cut bases of leaf sections in the assimilation chamber resulted in *ca* 60% inhibition of glycollate oxidase within 1 hr and 90% in 2 hr (data not shown). Following this treatment a similar distribution of ^{14}C-labelling was obtained as with *S*(+) HBA (Tables 1 and 2), consistent with the view that the inhibition of glycollate oxidase also results in an inhibition of photosynthesis. Accumulation of ^{14}C in glycollate was more pronounced, in accord with better penetration of the methyl ester or the higher concentration of inhibitor used, and incorporation of ^{14}C into sucrose was severely depressed.

Similar results were also obtained in experiments with leaf sections from plants where glycollate oxidase activity had been inhibited by feeding MeHBA to barley seedlings via the roots on the previous day.

The ^{14}C distribution pattern following treatment with 1.0 mM *R*(−) MeHBA is also given. In this case, partial racemization of the inactive *R*(−) enantiomer to the inhibitory *S*(+) enantiomer is suggested by somewhat increased incorporation into glycollate, and decreased incorporation into some other mettabolites, but these effects were less marked than for treatment with *S*(+) MeHBA.

Conclusions

These experiments show that metabolism via the glycollate pathway is strongly inhibited by the *S*(+) enantiomers of HBA and MeHBA. That this is due to specific inhibition of glycollate oxidase was substantiated by further experiments, including determination of the activities of glycollate pathway enzymes following exposure of barley leaf segments to MeHBA (Table 3).

The accumulation of ^{14}C in glycollate following treatment with active HBA compounds was accompanied by substantial decreases in the incorporated radioactivity in glycine and serine, consistent with their derivation from glycollate (Table 2). The contribution to serine synthesis from PGA via glycerate and hydroxypyruvate has, however, been stressed by several authors (see e.g. refs. [6, 29]). Its origin via a phosphorylated pathway involving 3-phosphohydroxypyruvate and phosphoserine is also a possibility [30–32]. The reduced incorporation of radioactivity into (and presumably reduced amount of) PGA after HBA treatment, does not preclude the possibility that labelling in serine arose from this source. However, the substantial decrease in [^{14}C]glycerate observed after HBA treatment tends to favour a flux of carbon predominantly from the glycollate pathway into serine. It thus seems probable that under normal (*in vivo*) conditions a flux of carbon via the glycollate pathway intermediates towards glycerate occurs, in line with the proposed functions of the photorespiratory pathway in carbohydrate metabolism.

The results obtained regarding net photosynthesis and glycollate metabolism following inhibition of glycollate oxidase with HBA compounds in the present studies are entirely in agreement with other recent reports. Sodium 2-hydroxy-3-butynoate has been used to inhibit glycollate oxidase in wheat leaf sections [7, 33], and the related ester butyl-2-hydroxy-3-butynoate has been used with isolated soybean leaf cells [8] and with intact sunflower leaves [34]. In all cases net photosynthesis was inhibited to varying extents. These data are in contrast to earlier findings [35] where net assimilation of $^{14}CO_2$ was increased after HPMS treatment of tobacco leaf discs, presumably owing to the inhibition of glycollate oxi-

Table 3. Effect on activities of glycollate pathway enzymes following greening of etiolated barley leaf segments in the presence of MeHBA

	Enzyme activity		
	Etiolated	Greened MeHBA absent	Greened MeHBA present
Glycollate oxidase	1.07	1.34	Negligible
Glutamate : glyoxylate aminotransferase	1.21	4.47	> 4.5
Serine : glyoxylate aminotransferase	1.21	1.65	1.04
Serine hydroxymethyl-transferase	0.05	0.06	0.08
Hydroxypyruvate reductase	4.9	7.3	5.9

1 g of etiolated 7-day-old barley leaf segments, *ca* 1 cm long, were floated on 20 ml 2.5 mM KH_2PO_4, pH 7.0, containing 1.5 mM MeHBA. Samples, in lidded Petri dishes, were incubated in darkness for 3 hr then illuminated (*ca* 4 klx) at 26° for 48 hr before enzyme estimation. Activities (μmol/min/g fr. wt) in extracts from etiolated leaves, and in leaf segments from etiolated shoots greened as above but not exposed to inhibitor are also shown.

dase and associated photorespiratory metabolism. Consistent with the present observations, large increases in the incorporation of $^{14}CO_2$ in glycollate, and concomitant decreases in glycine, serine, sucrose and phosphate esters [7] or decreases in serine, glycerate and starch, with negligible labelling of glycine [8], have been reported by other workers to be a consequence of inhibition of the glycollate pathway.

It has been suggested that these decreases in photosynthesis following chemical inhibition of glycollate pathway metabolism may be due to depletion of Calvin cycle intermediates [8]. Thus it has been shown [8; Kerr, M. W., unpublished] that HBA does not affect photosynthetic rate in the absence of oxygen. However, in its presence so much carbon from the Calvin cycle may be diverted into glycollate, which, owing to the inhibition of the Photorespiratory Carbon Oxidation route, cannot be returned to this cycle, that insufficient ribulose-bisphosphate is regenerated to maintain normal photosynthetic rates [8]. While our data obtained after glycollate oxidase inhibition by active HBA and MeHBA enantiomers are entirely consistent with this view, other interpretations are possible. Thus, the prevention of some suggested but unconfirmed function of glycollate metabolism, e.g. photorespiratory energy dissipation [36], or removal of excess reducing power [29], could also result in decreased photosynthesis. This might also occur as a result of the accumulated glycollate; it was observed that 7.4 mM glycollate caused *ca* 30% inhibition of $^{14}CO_2$ fixation by isolated spinach chloroplasts [37].

Effect on greening

It has been suggested that in greening tissue the glycollate pathway may make an appreciable contribution in the formation of chlorophyll [21–23] and chloroplast terpenoids [24]. The availability of a specific inhibitor of glycollate oxidase provided a useful tool to explore the contribution of glycollate-derived products to chloroplast pigment formation.

Experiments were carried out using etiolated leaf segments floated on the appropriate solution. Over a 48 hr period at 4 klx illumination untreated tissue typically formed *ca* 700 μg chlorophyll and gave an $A_{480}^{car.}$ of 35 on a g fr. wt basis. Though these amounts are only *ca* 70% those in the normal green leaf the method was convenient for presentation of inhibitors and substrates. Most of the pigment was formed over the first 30 hr illumination but amounts of both chlorophylls and carotenoids thereafter continued to increase slowly: a 48 hr illumination period was used routinely. The optimal age of seedlings for such greening experiments was 7 days from germination, when seedlings were 8.0–10.0 cm tall. Leaf segments were routinely floated on 2.5 mM potassium dihydrogen phosphate, pH 7.0, but pigment accumulation was more or less constant over the pH range 4–9 using acetate or Tris buffers as appropriate. Higher concentrations of buffer had a noticeable detrimental effect on pigment formation.

The effect of MeHBA on the greening of etiolated barley was followed by exposing leaf segments to various concentrations of a racemic mixture or enantiomeric forms of the inhibitor (Fig. 4). Chlorophyll formation was inhibited *ca* 65% by 0.75 mM *S*(+) MeHBA and *ca* 75% by 1.5 mM *S*(+) MeHBA. Though there was some inhibition (20 and 25%, respectively) by the same concentrations of *R*(−) MeHBA this was substantially less than for the *S*(+) enantiomer. The effect of the racemic (*RS*) mixture was consistent with the view that the lower activity of the *R*(−) enantiomer was due to racemization to the *S*(+) form since the inhibition due to *R,S*-MeHBA over a range of concentrations coincided with the inhibition due to *S*(+) MeHBA at half the concentration (Fig. 4). In these experiments, inhibition of carotenoid formation was similar to that observed for chlorophyll. Enantiomers of HBA behaved similarly though at comparable concentrations the *S*(+) enantiomer proved somewhat less

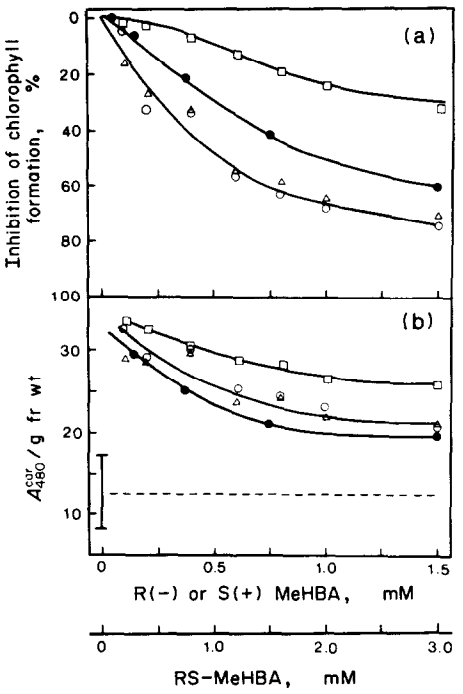

Fig. 4. Inhibition of pigment accumulation in illuminated etiolated barley leaf segments by enantiomeric forms and racemic mixture of MeHBA. 1 g of etiolated 7-day-old barley leaf segments, *ca* 1 cm long, were floated on 20 ml 2.5 mM KH_2PO_4, pH 7.0, containing *R*(−) MeHBA (□), *S*(+) MeHBA (△), or *R*, *S*-MeHBA (○) at the concentration indicated. Samples, in lidded Petri dishes, were incubated in darkness for 3 hr then illuminated (*ca* 4 klx) for 48 hr at 26° before pigment estimation. Chlorophyll accumulated in illuminated control samples was 730 ± 86 (n = 4) μg/g fr. wt. (---) Indicates mean ± s.d. (n = 4) for carotenoid in etiolated tissue. Data for an experiment with *R*, *S*-MeHBA under the same conditions except incubations were in conical flasks flushed with air containing 5% (v/v) CO_2 are also shown (●).

effective than *S*(+) MeHBA, the inhibition of chlorophyll formation by 0.75 mM *S*(+) HBA being only some 45%. This may be explained by the observation that racemization occurs to greater extent with HBA enantiomers than with those of MeHBA [Jewess, P. J., personal communication].

In an atmosphere containing 5% CO_2, where flux through the glycollate pathway should be depressed, pigment formation was still markedly inhibited by *R*, *S*-MeHBA though not to the same extent as in normal atmospheric CO_2 concentrations. Incubation at 25 instead of 4 klx had no effect on the inhibition by *S*(+) MeHBA.

Effect on enzymes of the glycollate pathway

That the site of action of MeHBA in greening barley was glycollate oxidase was confirmed by an assessment of the activities of glycollate pathway enzymes in leaf segments greened normally or in the presence of the inhibitor (Table 3). Since etiolated and green leaf tissues were used, enzyme activities are given on a fr. wt basis. Parallel determinations of protein by the Folin–phenol method showed that 1 g fr. wt green tissue was equivalent to *ca* 30 mg soluble protein, while etiolated tissue yielded *ca* half this amount. Compared with that in etiolated tissue, activity of all the enzymes increased on greening though in some cases the effect was marginal. The most marked increase in greened compared with that in etiolated tissue was for glutamate:glyoxylate aminotransferase. Activities in normal green tissue (not shown) were the same as those for greened leaf segments.

The data obtained confirmed that glycollate oxidase activity was negligible following treatment with MeHBA. There was also decreased activity for serine:glyoxylate aminotransferase, though in contrast activity of glutamate:glyoxylate aminotransferase was enhanced. These latter effects of MeHBA, which were not explored further, may be indirect, and occur as a result of changed amounts of metabolites acting as activators or inhibitors of these aminotransferases.

For greening experiments with etiolated leaf segments, incubation for 3 hr with 1.0 mM MeHBA was sufficient to obtain *ca* 80% inhibition of glycollate oxidase (Fig. 3). Enzyme levels were not restored during a subsequent 24 hr illumination. In agreement with other workers ([2, 7] but cf. ref. [10]), we found that total inhibition of glycollate oxidase in tissues is not achieved, possibly because the accumulation of glycollate protects some of the FMN cofactor of the enzyme. As Fig. 3 additionally shows, glycollate oxidase in normal green seedlings was similarly inhibited within 24 hr by exposure to MeHBA via the roots. The lower MeHBA concentration if supplied over the whole period of the experiment caused 80% inhibition of glycollate oxidase.

The correlation between inhibition of glycollate oxidase and of chlorophyll formation was also confirmed by experiments with etiolated seedlings exposed to a range of MeHBA concentrations (Fig. 5). This shows the maximum inhibition of chlorophyll

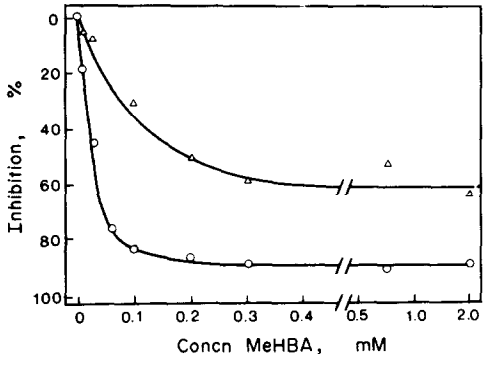

Fig. 5. Effect of increasing MeHBA concentration on inhibition of chlorophyll accumulation and glycollate oxidase in greening barley seedlings. 7-day-old barley seedlings, with the roots immersed in nutrient solution containing MeHBA at the concentration indicated were illuminated (*ca* 4 klx) at 26° for 24 hr before estimation of chlorophyll (△) and glycollate oxidase (○) in the leaves. Enzyme activity in untreated controls was 1.6 μmol/min/g fr. wt; chlorophyll was 1000 μg/g fr. wt.

formation (60%, cf. Fig. 4) is produced by 0.3 mM MeHBA, an inhibitor concentration which gives *ca* 90% inhibition of glycollate oxidase. The greater potency observed when MeHBA is taken-up via the roots of intact seedlings compared to treatment of leaf segments (Fig. 4) implicates the roots as the site of the esterase catalysing hydrolysis of the ester to the active HBA. Glycollate oxidase activity in leaf extracts is thus unaffected by the addition of MeHBA to the assay mixture, as with *P. sativum* [2]. As with other species [2], glycollate oxidase was never completely inhibited by MeHBA supplied via the roots.

Attempts to restore greening after MeHBA treatment

In previous work with isonicotinyl hydrazide [38], which inhibits pyridoxal phosphate-requiring enzymes, supplementation with later intermediates in the pathway was found to alleviate the inhibition of greening. Similar attempts were made to restore pigment formation in MeHBA-inhibited barley. However, none of the intermediates of the glycollate pathway, or pyruvate, would restore greening other than marginally. Sucrose, supply of which restores greening in plant tissues where this process is inhibited in the presence of DCMU [39, 40], was also ineffective.

The most effective attempts to restore greening following MeHBA treatment all involved glycine in combination with some other metabolite. With formate, glycine restored carotenoid by 60% but chlorophyll by only 20%. Combinations of glycine, succinate and either pyruvate or serine, and of glycine with glutamate were markedly more effective and in the case of the two last supplementations normal chlorophyll and carotenoid concentrations were fully restored. The role of glycollate in C_1 metabolism is well established [41] and the efficacy of glycine and formate suggests that formation of endogenous serine within the tissue may be more effective than that supplied exogenously. The restoration of greening in the other cases can be interpreted as the need for glycine and succinate as precursors of aminolaevulinic acid [23] and of serine or pyruvate as precursors of chloroplast terpenoids [24] in greening tissue. The success of glycine in combination with glutamate is in accord with the suggested role of the latter C_5 compound in chlorophyll synthesis in some photosynthetic tissues [42].

Conclusions

These experiments convincingly demonstrate that specific inhibition of glycollate oxidase by the stereospecific *S*(+) enantiomer of HBA is accompanied by a parallel inhibition of chloroplast pigment formation. The failure to restore greening other than by some combinations of metabolites may suggest a role for glycollate in chloroplast development separate from its initiation of the photorespiratory pathway (see e.g. ref. [8]), or that in the absence of the latter during greening some irreversible damage, possibly photoinhibition [43], occurs.

EXPERIMENTAL

Growth of seedlings. Seeds of *H. vulgare*, variety Mazurka, were obtained from The Welsh Plant Breeding Station, Aberystwyth, and grown in Vermiculite or soil in an environmental growth chamber. Etiolated seedlings were grown in darkness at 25°. For normal (green) seedlings growth was under a 14 hr day regime (day temp. 26°, night temp. 20°) with fluorescent and tungsten lights providing 8 klx at the leaf surface.

Greening studies. A method based on ref. [38] was routinely used. Etiolated primary leaves, usually 7 days from germination, were cut into segments *ca* 1 cm long and a known fr. wt (*ca* 1 g) was floated on 20 ml H_2O or a soln containing buffer, inhibitor and/or metabolite as required, in lidded plastic Petri dishes. In some experiments etiolated whole or excised seedlings or longer (etiolated) leaf sections were used. Whole seedlings were gently uprooted and the roots washed, while excised seedlings were cut just above ground level then stood in conical flasks containing H_2O or 0.05 (w/v) plant nutrient soln (Phostrogen Ltd., Clwyd), with inhibitor as necessary. Sections (normally five) of the primary leaves of etiolated barley, 3–4 cm long, were obtained by excising the leaf tip and lower leaf and were stood in small open-topped glass containers with the lower cut ends immersed in 2 ml H_2O or soln. Operations were carried out in darkness or under dim light conditions.

Some expts necessitated the return of samples to darkness for various incubation periods before illumination whereas in others samples were illuminated directly after preparation. Illumination was carried out in a controlled environment cabinet at 26° with continuous light from a standard combination of four fluorescent tubes (cool white) and two 60 W tungsten bulbs, giving a light intensity of *ca* 4 klx at the leaf surface. The period of illumination was routinely 48 hr. After illumination, leaf material was quickly dried and if necessary weighed before storage in the dark at 4° to await pigment extraction and estimation. Storage for several hours had negligible effects on estimated amounts of pigments. Soln pH was adjusted to pH 7.0 before illumination using dil. KOH or HCl, and routinely checked after illumination. Incubations where the pH had changed markedly were discarded.

Pigment extraction and estimation. Leaf material was extracted in Me_2CO [38] based on the method of ref. [44], and chlorophyll was determined using the revised equations given in ref. [45]. Carotenoids were estimated at 480 nm and the *A* corrected for chlorophyll using the equation:

$$A_{480}^{car.} = A_{480} + 0.14A_{664} - 0.568A_{647}.$$

This equation is based on that in ref. [46] but is modified to incorporate revised extinction coefficients for chlorophylls *a* and *b* [45], and for chlorophylls at 480 nm [47].

$^{14}CO_2$ assimilation. The apparatus and sample chambers for supplying a $^{14}CO_2$–air mixture to leaf sections were based on those used by workers at Rothamsted Experimental Station (see e.g. refs. [7, 12, 26, 48, 49]). Full details are given in ref. [50].

Primary leaves of *ca* 2-week-old normally grown barley seedlings (2 leaf stage) were used. They were detached at the stem from seedlings that had been kept in darkness for the preceding 18 hr and placed in a wire frame in the leaf chamber. The total area of leaf material directly exposed to incident light (5 or 25 klx) was *ca* 10 cm^2 (4 leaf sections; *ca* 250 mg fr. wt). The frame was then placed in the leaf chamber so that the cut bases of the leaf sections were immersed in 1 ml H_2O or appropriate inhibitor soln. After equilibration with normal air, leaf sections were exposed to air, humidified at 20°, containing 300 μl $^{14}CO_2$/l. (0.075 or 1.87 Ci/mol) at a flow rate of 0.8 l/min. At the end of the

selected period of photosynthesis in $^{14}CO_2$–air, the tissue was rapidly killed by plunging the complete frame with leaf sections into a tube containing *ca* 20 ml boiling 80% (v/v) aq. EtOH. Boiling was continued for 2–3 min by immersion of the tube in a water bath at 80° before cooling to room temp.

Preparation of ^{14}C *assimilate extracts.* Leaf sections were homogenized in 80% EtOH and the residue after centrifugation exhaustively extracted with further vols. of boiling EtOH and H_2O. The bulked extracts were reduced to dryness under vacuum at 35°. Less than 1% of the ^{14}C was present in the distillate and negligible amounts of ^{14}C-material were lost by this treatment. The methods of refs. [51, 52] were used to further separate the residue into H_2O-soluble and lipid components. The H_2O-soluble components were further separated into basic, acidic and neutral fractions by ion exchange chromatography on columns (6 × 0.9 cm) of Amberlite cg 120 (H^+) and Deacidite FF (formate form). 99% of added radioactivity was recovered in the separated fractions.

Individual compounds in the H_2O-soluble fraction were separated by 2D-TLC [12, 48, 49, 53] using the solvents given in ref. [54]. H_2O-soluble extract containing usually 100 000 dpm of ^{14}C assimilate (up to *ca* 50 μl., equivalent to *ca* 12 mg fr. wt leaf) was applied as a spot 2.5 cm diagonally from a corner of the plate, using a μl. syringe and drying between successive (*ca* 2μl.) applications with a cool air stream. Plates were developed at *ca* 18° in the first direction with PrOH–NH_3–H_2O (6:3:1) containing 0.2% (w/v) Na_2EDTA until the solvent front was within *ca* 2 cm of the top of the plate (3–4 hr). Plates were then dried vertically in a cool air stream until excess solvent had evaporated, then horizontally for several hours or overnight, before developing in the second direction (for *ca* 5 hr) with *n*-propyl acetate–90% HCO_2H–H_2O (11:5:3) before plates were then thoroughly dried as before. Radioactive areas were located by autoradiography with Kodak X-ray film. Compounds were identified using published R_f values [53–55] and by cochromatography.

Assay of radioactivity. Radioactivity was assayed by liquid scintillation in N260 fluid and all counts were corrected for quenching. The counting efficiency was better than 90%. Areas of the TLC plates corresponding to radioactive compounds were first treated with collodion [51] before removal for radioassay in NE260.

Enzyme assays. A known wt of leaf material (0.2–1.0 g) was thoroughly homogenized at *ca* 4° in 10 ml 0.1 M Tris–HCl, pH 8.2, by several 5–10 sec bursts with an Ultra-Turrax homogenizer. The homogenate was centrifuged at *ca* 2000*g* for 15 min and the resulting supernatant used.

Glycollate oxidase assays were carried out in an O_2-electrode as in ref. [2], except that the sodium glycollate concentration was 1 mM, generally using 1 ml of extract containing 1–2 mg protein, although this varied depending on activity. Enzyme units (μmol O_2 converted/min) calculated on a fr. wt or protein basis gave comparable results, hence for most expts enzyme units are quoted as μmol O_2/min/g fr. wt.

Glutamate:glyoxylate aminotransferase [56, 57] and serine hydroxymethyltransferase [59] were assayed by radiochemical methods, and serine:glyoxylate aminotransferase [57, 58] and hydroxypyruvate reductase by spectrophotometric methods. For the latter the reaction mixture in a total vol. of 3.0 ml at 25° contained: Hepes buffer (pH 7.0) 200 μmol; NADH, 0.2 μmol; and 0.2 ml of plant extract. A slow endogenous rate was measured over a 90 sec period before starting the reaction by the addition of 2 μmol hydroxypyruvate. In all cases activities were calculated as μmol/min/g fr. wt, using for the spectrophotometric assays a $\epsilon_{340\,nm}$ of 6.22×10^3 l/mol/cm for NADH.

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