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GLYCINE METABOLISM AND CHLOROPHYLL SYNTHESIS IN BARLEY LEAVES

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; leaves; glycine; α-hydroxypyridine methane sulphonic acid; δ-aminolaevulinic acid; chlorophyll.

Abstract—α-Hydroxypyridine methane sulphonic acid (HPMS), isonicotinyl hydrazide (INH) and nialamide inhibit chlorophyll synthesis in etiolated barley leaves exposed to light. HPMS lowered the rate of protochlorophyllide regeneration but had little effect on the synthesis of protochlorophyll (P630) from exogenous δ-aminolaevulinic acid (ALA). The addition of glycine to HPMS treated leaves partially overcame the inhibition of chlorophyll synthesis. Glycine-[^{14}C] was readily incorporated into ALA in dark-grown leaves. HPMS treatment increased the sp. act. of ALA in leaves fed glycine-[^{14}C]. Glycollate oxidation was lower in extracts from HPMS treated leaves. Plants may therefore have two pathways for ALA production with the glutamate pathway becoming more important in conditions where photorespiration is high.

INTRODUCTION

In the last few years a number of reports have appeared suggesting that higher plants may have a novel system for the synthesis of δ-aminolaevulinic acid (ALA) [1–7]. This work has largely been based on relative isotope incorporation studies into ALA accumulated in the presence of laevulinic acid (LA), a competitive inhibitor of ALA-dehydrogenase [8]. Such studies indicate that glutamate-[^{14}C] is a better source of carbon and nitrogen than glycine-[^{14}C]. Recent work however, has shown that glycine levels [9] in ageing and greening barley leaves, unlike other amino acids [10], correlate well with the ability to synthesize chlorophyll [11]. Also, since glycine-[^{14}C] is rapidly turned over in higher plant leaves [12], conclusions based on relative incorporation efficiencies should be viewed with some care. The present investigation reports the effect of induced low endogenous glycine levels on chlorophyll and ALA synthesis in barley leaves.

RESULTS

Glycine-[^{14}C] incorporation into ALA

In a number of experiments on the fate of glycine in etiolated and greening barley leaves it was found that glycine-[^{14}C] feeding resulted in radioactive ALA. In a typical experiment the ALA recovered from aqueous extracts of leaf sections from 7-day-old etiolated leaves given a 2 hr incubation in glycine-[^{14}C] contained nearly 0.5% of the total radioactivity present in this fraction. Illumination for 4 hr of the leaves before incubation in glycine-[^{14}C] lowered the incorporation into ALA to 0.1%. The ALA recovered from leaf sections incubated in glycine-[^{14}C] in the presence of LA (10^{-2} M) contained substantially higher radioactivity with often as much as 3% of the total radioactivity in the aqueous fraction. These results were made more interesting since no incorporation of glutamate-[^{14}C] into ALA was achieved in tissue not fed LA. Attempts were made to measure the ALA pool in 'non-fed' LA leaves. This proved difficult even when up to 1000 leaf segments were used. The use of carrier ALA indicated that the amount of endogenous ALA was 10^{-8}–10^{-10} mol/100 segments.

Table 1. The effect of glycollate oxidation inhibitors on chlorophyll and carotenoid production in barley leaves

	Total chlorophyll		Carotenoids	
Treatment	nmol/g fr. wt	% inhibition	*A* units/g fr. wt	% inhibition
INH				
10^{-2} M	149	66.2	14.3	16.3
10^{-3} M	287	35.3	15.0	12.3
10^{-4} M	430	2.8	17.3	−1.2
0	443		17.1	
Nialamide				
10^{-2} M	166	60.6	13.9	20.1
10^{-3} M	272	36.0	13.8	20.7
10^{-4} M	437	−2.9	17.7	1.7
0	425		17.4	
HPMS				
10^{-2} M	289	38.9	15.4	17.2
10^{-3} M	352	25.5	16.1	13.4
10^{-4} M	373	21.3	16.2	12.9
0	473		18.6	

7-day-old dark-grown leaves were cut 8 cm below the tip and incubated for 100 min in the dark in INH nialamide or HPMS. Feeding was continued in the light for 22 ± 1 hr (separate controls are given) after which pigments were extracted and assayed.

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Table 2. The effect of HPMS on chlorophyll production in barley leaves

HPMS (M)	Total chlorophyll nmol/g fr. wt	Total chlorophyll % inhibition
10^{-1}	6.1	92.9
10^{-2}	59.6	30.7
10^{-3}	61.4	28.6
10^{-4}	69.7	19.0
10^{-5}	82.0	4.7
0	86.0	

7-day-old dark-grown leaves were cut 8 cm below the tip and fed HPMS (pH 5) for 5 hr in the light after which chlorophyll was determined.

Table 3. Protochlorophyllide regeneration and P630 production form ALA in HPMS treated barley leaves

HPMS	P652 regeneration (Δ652 change × 10^3/100 sec)	P630 production from ALA (Δ630 change × 10^3/100 sec)
10^{-1}M	·0	2
10^{-2}M	12	6
10^{-3}M	17	5
10^{-4}M	38	6
0	42	6

In vivo rates were determined in the penultimate 2 cm of 7-day-old dark-grown barley leaves.

Effect of inhibitors of glycollate metabolism on chlorophyll formation

INH, HPMS and nialamide were all tested for their effects on chlorophyll synthesis in barley seedlings. The results (Table 1) show that all 3 compounds were capable of inhibiting chlorophyll synthesis. At higher concentrations (10^{-2} and 10^{-3}M) INH and nialamide reduced chlorophyll synthesis *ca* 35 and 60% respectively. At lower concentrations (10^{-4}M) these two compounds proved ineffective. HPMS, on the other hand, whilst not as efficient an inhibitor as INH or nialamide, was still very effective at lower concentrations.

Further experiments with the nicotinyl hydrazines indicated that their uptake was relatively slow and that they, besides inhibiting chlorophyll production, altered amino acid metabolism quite considerably (they induced a decrease in aspartate and increased arginine, lysine and histidine).

The effect of HPMS on chlorophyll synthesis

In previous experiments incubation times had been relatively long with the induction of possible artefacts. The results for shorter term incubation periods on chlorophyll and carotenoid production are given in Table 2. Even after 5 hr incubation, HPMS at 10^{-1}M virtually suppressed all chlorophyll synthesis.

The effect of HPMS on P650 production from exogenous ALA

To determine whether HPMS was being an effective chlorophyll inhibitor by limiting the supply of endogenous ALA the regeneration of protochlorophyllide (P650) was measured in treated dark-grown leaves given a brief light period. The results (Table 3) show that at all HPMS concentrations there was a reduction in the rate of regenerated P652–with 100% inhibition observed at 10^{-1}M HPMS. HPMS, on the other hand, had little effect on the rate of P630 formation from exogenous ALA in the dark (Table 3).

The effect of glycine and glyoxylate on HPMS inhibition of chlorophyll synthesis

At relatively low concentrations, HPMS may have little effect on cell metabolism other than the inhibition of glycollate oxidation and chlorophyll synthesis. If glycollate and chlorophyll synthesis are closely related it may be possible to overcome the HPMS inhibition of chlorophyll synthesis by feeding substrates formed from glycollate. Seven-day-old leaves were cut 8 cm below the tip, fed HPMS (10^{-3}M, pH 7.5) for 17.5 hr in the dark and after transferring to 10^{-3}M glycine given 5 hr light. In leaves fed HPMS only, 84% chlorophyll was synthesised compared to control leaves. In the HPMS plus glycine treatments the leaves produced nearly 99% chlorophyll compared to controls fed glycine alone. Glyoxylate however, did not overcome the HPMS inhibition of chlorophyll synthesis.

The effect of HPMS on ALA, glycine and chlorophyll synthesis

The effect of HPMS in combination with LA on ALA accumulation, glycine levels and chlorophyll production, in segments from 8-day-old dark-grown barley leaves, was investigated. The results (Table 4) indicate that HPMS (at 10^{-2}M) is as powerful an inhibitor of

Table 4. The effect of HPMS in the presence of laevulinic acid on ALA, glycine and chlorophyll synthesis

Treatment	Total chlorophyll nmol/g fr. wt	Total chlorophyll % inhibition	Glycine nmol/g fr. wt	Glycine % inhibition	ALA nmol/g fr. wt	ALA % inhibition
HPMS (10^{-2}M) + LA (5×10^{-2}M)	29.6	48.2	351	60.5	86.6	23.9
HPMS (10^{-2}M)	35.2	39.8	340	58.6	0	
LA (5×10^{-2}M)	36.5	36.0	510	37.9	112	
H_2O	57.0		580		0	

8-day-old dark-grown leaves were cut 4 cm below the tip and fed HPMS and LA together and separately at pH 5 for 6 hr in the light.

Table 5. The effect of HPMS on glycine-[^{14}C] incorporation into ALA in greening barley leaves

Treatment	Total uptake (% dpm supplied)	sp. act. Glycine	sp. act. ALA
HPMS (10^{-2} M)	5.74	1300	381
H_2O	9.88	899	213

8-day-old dark-grown leaves were cut 8 cm below the tip fed HPMS or DW for 16 hr in the dark, and transferred to laevulinate (5×10^{-2} M) and glycine-[U-^{14}C] (2.5 μCi; sp. act. 114 mCi/mmol) for 16 hr in the light. Sp. act. is calculated as:

$$\frac{\text{dpm (glycine or ALA)}}{\text{Total dpm uptake} \times \text{mmol (glycine or ALA)} \times \text{fr. wt.}}$$

chlorophyll synthesis as LA at 5× the concentration of HPMS. The two inhibitors in combination inhibited chlorophyll by 48% over 10% more than with the inhibitors alone. HPMS lowered the accumulation of ALA in the presence of LA by some 24% while glycine levels were reduced by 31%. Glycine in HPMS treated tissue was also significantly lower than in controls.

The effect of HPMS on glycine-[^{14}C] incorporation into ALA

Glycine-[^{14}C] (2.5 mCi, sp. act. 7.2 mCi mol) was fed to segments of 8-day-old dark-grown leaves in the presence of HPMS. After 16 hr dark incubation the leaves were transferred to LA (5×10^{-2} M) for 6 hr in the light. The sp. act. of the glycine remaining in the tissue, and that of the ALA accumulated, were determined (Table 5). The sp. act. of glycine in HPMS treated leaves was 45% higher than that of controls. Similarly the sp. act. of accumulated ALA was higher in the HPMS treatments.

Glycollate oxidation

Glycollate oxidase activity was measured in extracts of ageing dark-grown leaves given a light treatment of up to 48 hr. Maximum rates of glycollate oxidation of 4.5 ng atoms O_2/mg protein/hr were found in extracts of 8-day-old etiolated leaves. By day 9 the glycollate oxidation rates dropped nearly 50% and thereafter remained similar up to day 15. Illumination of the intact leaves induced a sharp rise in oxidation rate, detectable in leaf extract within 2 hr light as an increase of 6.7% in 7-day-old dark-grown leaves (Table 6). At this age maximum rates of oxidation were found after 24 hr light. In the older leaves (up to 10 day) oxidase activity increased on illumination. In 15-day-old material no increase in activity was observed on exposure to light.

To confirm that HPMS lowered glycine production *in vivo* leaves in the presence of the inhibitor were fed glycollate-[^{14}C] and the sp. act. of the glycine formed determined. Eight-day-old dark-grown leaves were cut 8 cm below the tip, fed HPMS (10^{-2} M) for 16 hr in the dark and transferred to glycollate-[1-^{14}C] (2.5 μCi) for 6 hr in the light. The glycine was then extracted and after purification assayed for radioactivity. The sp. act. dpm glycine/total dpm uptake × mmol × fr. wt) of glycine recovered from HPMS treated leaves was 292 compared to a sp. act. of 595 for the glycine from control leaves.

Table 6. Glycollate oxidation activity in protein extracts from illuminated etiolated barley leaves

Leaf age (days in dark)	Light (hr)	Oxygen uptake (ng atoms/mg protein/hr) Light	Dark control	% increase in light
7	2	3.64	3.41	6.7
	5.5	4.26	3.70	15.1
	24	7.14	4.52	58.0
	30	9.93	4.15	139.3
	48	8.62	2.47	248.9
9	5.5	5.12	2.46	108.1
	24	6.70	2.43	275.7
10	5.5	2.39	2.42	−1.3
	24	2.12	2.38	−11.0

Seedlings were grown in the dark for 7 to 10 days and exposed to light for various times and protein extracted in cold Tris–HCl (0.1 M, pH 8). After centrifugation oxygen uptake was measured in an O_2 electrode before and after the addition of 0.1 μmol glycollate in 0.1 ml Tris–HCl.

DISCUSSION

Glycollate oxidation in barley leaves appears to be the major route for the synthesis of glycine particularly when chlorophyll production becomes exponential in light treated leaves. Glycollate oxidase activity declines rapidly in leaves after 8 days dark-growth and follows the rapid diminution in glycine and the ability to produce chlorophyll which occurs at this time. HPMS, an inhibitor of glycollate oxidation, [13, 14] reduced glycine levels in barley leaves and proved to be an efficient inhibitor of chlorophyll synthesis. Other inhibitors, known to interfere with glycollate metabolism [15, 16, 17] also inhibited chlorophyll production to a marked degree. HPMS appeared to act at a point before ALA synthesis, and from glycine-[^{14}C] incorporation studies this was at a step in the production of endogenous glycine. HPMS (10^{-2} M) induced a reduction in ALA levels in the presence of LA, by 24% and increased the sp. act. of ALA by 79% after glycine-[^{14}C] feeding. This correlates well with the inhibition of glycollate oxidation and hence the low levels of glycine in HPMS treated leaves thereby making available more substrate-[^{14}C] (glycine) for the synthesis of ALA. This is supported by the evidence that HPMS increased the sp. act. of the glycine pool(s) after glycine-[^{14}C] feeding by 45% and that exogenous glycine can partially overcome the HPMS inhibition of chlorophyll synthesis. There is then much circumstantial evidence to favour glycine as a precursor of ALA (under certain physiological conditions) in barley. This evidence is summarised as follows: (1) Glycine levels reach their maxima in 7-day-old dark-grown leaves [9], one day in advance of the maximum level of protochlorophyllide [11]. (2) Glycine is the only amino acid to show a rapid decline in the period 7 to 10 days [9] during which time protochlorophyllide also declines [11], ALA synthesis rates are reduced [18] and there is an increased loss of ability to form chlorophyll on illumination [11]. (3) Glycine turnover is rapid in the dark [12] and in the absence of any major demand for ALA, substantial amounts of glycine are deaminated [19]. Light induces a demand on precursors for ALA formation when glycine deamination is inhibited. (4) Light regulates chlorophyll

[19], protohaem [20], protochlorophyllide [21], ALA and glycine turnover [12]. (5) In the light glycine is formed from carbon skeletons derived, in young leaves, from photosynthetically formed glycollate. The amino group of glycine is derived mainly from glutamate in microsomes which appear to be the principal site of glycine formation [23]. The amino group of glutamate derived from NH_4^+, is ultimately dependent on light-mediated nitrate and nitrite reduction in barley leaves [24]. All of the glycine molecule may therefore be formed from light dependent or light-stimulated reactions. (6) Teleologically, glycine is ideally suited to ALA formation in that its synthesis is closely linked to photosynthesis.

Appreciable quantities of glycine-[^{14}C] were incorporated into ALA in 7-day-old leaves in the dark, whereas little or no incorporation of labelled glutamate was found. It would appear that there is an active system for the conversion of glycine-[^{14}C] to ALA in dark-grown barley leaves. It may be that in the light, the glycine → ALA pathway becomes less important to one derived from glutamate. This may be a response peculiar to plants which, unlike animals, photorespire. With the loss of glycollate to CO_2 and increased demands for peptide synthesis, glycine pools are under stress and may be unable to meet demands for ALA and chlorophyll synthesis. Porra and Grimme [25] report that glycine-[2-^{14}C] was readily incorporated into ALA in greening *Chlorella* cultures under conditions of nitrogen starvation. Perhaps under these circumstances glycine incorporation makes a more important contribution than glutamate.

Plants may possess two pathways for the synthesis of ALA. One of these is similar to that found in animal and bacterial systems and utilises glycine particularly in plants grown in the dark. The second, which has developed in the course of evolution, avoids glycine under conditions of high photorespiration and plays a major role in the synthesis of ALA in the light as indicated by glutamate-[^{14}C] studies [3].

EXPERIMENTAL

Plant material and chemicals. Barley seed (*Hordeum vulgare*, cv Proctor) were imbibed for 16 hr, sown in vermiculite, and grown in the dark at 26°. Radioactive compounds (Radiochemical Centre, Amersham) were further purified by high voltage electrophoresis before use. HPMS was obtained from Fluka Chemicals.

Extraction and purification. Amino acids and ALA were exhaustively extracted in a hot (70°) aq. EtOH series. After reducing the vol. *in vacuo*, glycine and ALA were purified by high voltage zone electrophoresis (formate–acetate, pH 1.95 at 85 V/cm, for 28 min).

Quantitative assays. Chlorophyll was determined in 80% aq. Me_2CO [26] and ALA, after purification, as the Ehrlich product [27]. Glycine measurements were made using a ninhydrin method [28]. Soluble protein was determined as a complex with xylene G [29]. Measurements of protochlorophyllide regeneration and P630 synthesis from ALA were as previously described [19].

Radioassay. Radioactivity was determined by liquid scintillation in toluene with PPO and POPOP.

Glycollate oxidation. Protein extracts in Tris–HCl buffer, (0.1 M pH 8) were assayed for oxidase activity using polarographic techniques.

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