Phytochemistry, 1978, Vol. 17, pp. 671–674. Pergamon Press Printed in England.

EFFECT OF 2,2′-BIPYRIDYL ON PORPHYRIN SYNTHESIS IN ETIOLATED AND LIGHT-TREATED BARLEY LEAVES

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(*Received* 10 *November* 1977)

Key Word Index—*Hordeum vulgare*; Gramineae; barley; chlorophyll; haem; 2,2′-bipyridyl; δ-aminolaevulinic acid.

Abstract—The effects of 2,2′-bipyridyl on porphyrin formation differed in illuminated and dark-treated barley leaves. In the dark, bipyridyl treatment increased photoconvertible protochlorophyllide (Pchlide, P650) and decreased the protohaem content. The increase in Pchlide could not be wholly accounted for by a diversion of 'substrate' from protohaem synthesis. The rate of Pchlide regeneration was slightly higher in chelator treated leaves which suggests increased δ-aminolaevulinic acid (ALA) synthesis. Only small quantities of Mg-protoporphyrinmonomethylester (Mg-protoME) were detected in etiolated leaves treated with bipyridyl in the dark. Protochlorophyll (P630) synthesis from exogenously supplied ALA was lower in the chelator treatments. The results suggest that only when *substantial* quantities of ALA are being utilized in dark-grown leaves does a 'metal' become limiting in the bipyridyl treated leaves. In the light, bipyridyl inhibited chlorophyll synthesis, again suggesting that when substantial amounts of ALA were being utilized a 'metal' becomes rate limiting. Bipyridyl treatment also inhibited ALA production in light-treated leaves. The incorporation of glycine-[^{14}C] into ALA in the presence of bipyridyl was severely restricted compared to the incorporation of glutamate-[^{14}C]. The data suggest two pathways for ALA synthesis; the classical ALA-synthetase which utilizes glycine and is operative in dark-grown leaves and a second enzyme system, which uses glutamate, and is of quantitative importance in the light.

INTRODUCTION

Mammals, birds, bacteria and fungi form ALA largely or exclusively through the ALA-synthetase (glycine-succinyl-CoA condensation) reaction. Convincing evidence for a similar pathway in higher plants is lacking [1] and alternative reactions utilizing a derivative of glutamate in ALA formation have been described [2]. However, circumstantial evidence implicating glycine in the synthesis of ALA in barley leaves in the dark has been presented [3]. There is an extensive literature on the regulation of ALA and porphyrin synthesis by iron and haem in bacteria and animals. If the formation of ALA in higher plants is controlled by an enzyme other than the classical ALA-synthetase, then it may be that the role of iron and haem in ALA formation is different in plants from other organisms. Several effects of iron chelators on etiolated bean leaves [4] and wheat leaves [5] have been described which support this possibility. However, other workers [6] have postulated that haem regulation of porphyrin synthesis is greening barley leaves may be similar to that proposed for animals and bacteria. The following paper reports the use of bidentate chelators on ALA and porphyrin production in both dark-grown and greening barley leaves.

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† Please send reprint requests to AKS. Abbreviations: ALA, δ-aminolaevulinic acid; LA, laevulinic acid; Pchlide (P650), photoconvertible protochlorophyllide; Proto IX, protoporphyrin IX; Copro, coproporphyrin; Mg-protoME, magnesium protoporphyrin IX methyl ester; PBG, porphobilinogen.

RESULTS

Effect on porphyrins in dark-grown barley leaves

Seven-day-old 6-cm tops from dark-grown primary leaves were fed 2,2′-bipyridyl for 21 hr in the dark and then exposed to light for 3 min to convert Pchlide, P650 to chlorophyll (-ide, P680). Protohaem and photoconverted Pchlide were determined (Table 1). Bipyridyl lowered protohaem by up to 55% (10 mM bipyridyl) but increased the amount of Pchlide by 68% (1 mM bipyridyl). *In vivo* spectrophotometry of bipyridyl-fed leaves failed to show any chlorophyll intermediates as found in chelator-treated bean leaves [4]. Extensive *in vivo* studies with 6, 8, 9 and 10-day-old dark-grown leaves fed bipyridyl (or *o*-phenanthroline) for 4–24 hr, with and without vacuum infiltration, at chelator concentrations

Table 1. The effect of bipyridyl on porphyrin production in dark-grown leaves

Bipyridyl concentration (mM)	Photoconvertible protochlorophyllide nmol/g/fr.wt	% promotion	Protohaem nmol/g/fr.wt	% inhibition
10	10.65	47.5	2.25	55.4
1	12.13	68.0	3.65	27.7
0.1	10.9	50.9	4.06	19.6
0.01	7.45	3.2	4.58	9.3
0.001	7.15	−1.0	4.73	6.3
0	7.22		5.05	

Six-cm tops from 7-day-old dark-grown barley leaves were incubated in bipyridyl, pH 7.5, 21 hr in the dark. Photoconvertible protochlorophyllide and protohaem were then determined.

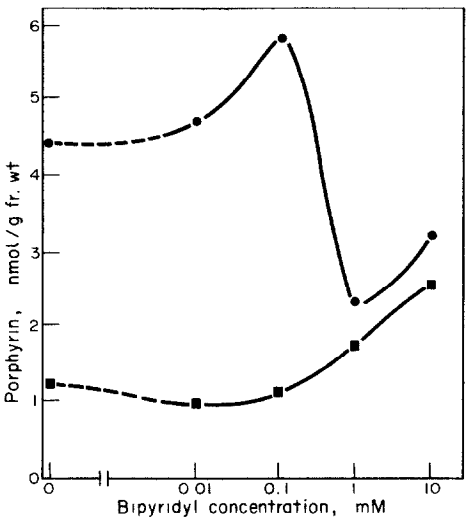

Fig. 1. Magnesium protoporphyrin IX methyl ester and protochlorophyllide production in leaves treated with bipyridyl. Six-cm tops from 8-day-old dark-grown barley leaves were fed bipyridyl (pH 7.5) for 20 hr in the dark, after which, protochlorophyllide and Mg-proto ME were determined. ■—■, Mg-protoME. ●—●, protochlorophyllide.

of 0.01 to 10 mM, also failed to show an increase in any chlorophyll precursor other than protochlorophyllide. Porphyrins from bipyridyl-fed leaves were partially purified by differential solubility in HCl. Copro and proto IX, in both chelator-fed and control leaves, were present only in trace amounts. Only the final 5N HCl fraction had an absorbance spectrum similar to that described for Mg-ProtoME (or its acid derivatives) [7]. The results (Fig. 1) show that, in bipyridyl concentrations of up to 0.1 mM Pchlide increased without any change in Mg-ProtoME. Only at 1 mM bipyridyl, when Pchlide fell by 50%, did Mg-ProtoME increase. However, the sum of the Pchlide and Mg-ProtoME concentration at 1 mM bipyridyl was 4.1 nmol/g fr. wt, 31% lower than the sum of the two porphyrins in the control treatment.

Table 2. The effect of bidentate iron chelators on porphyrin production in leaves in the light

Treatment (mM)	Protohaem nmol/g/fr. wt	Protohaem % inhibition	Chlorophyll nmol/g/ft. wt	Chlorophyll % inhibition
Bipyridyl				
10	2.2	43.7	45.0	83.4
5	2.06	47.3	63.1	76.6
2.5	2.22	43.2	104	61.5
1	2.3	41.2	141	48.1
0.5	3.15	19.4	200	26.2
o-Phenanthroline				
10	2.32	40.7	125	54.0
5	3.0	23.3	166	38.7
2.5	3.07	21.5	174	35.8
1	4.01	+2.5	181	33.2
0.5	5.22	+33.5	223	17.6
Control	3.91		271	

Six-cm tops from 8-day-old dark-grown barley leaves were fed the chelator for 1 hr in the dark and then for 15 hr in the light before haem and chlorophyll were determined.

Table 3. The effect of bipyridyl on δ-aminolaevulinic acid accumulation in the presence of laevulinic acid

Bipyridyl concentration (mM)	δ-aminolaevulinic acid nmol/g/fr. wt	% inhibition
10	137	27.2
1	101	46.1
0.1	56	70.2
0	188	

Seven-day-old dark-grown barley leaves were cut 4 cm below the tip and the top 2 cm discarded. The remaining segments were incubated in bipyridyl and LA (5×10^{-2} M) in K–Pi buffer, pH 7.5 for 4 hr in the light. ALA was purified by HV electrophoresis before estimation.

Effect of bipyridyl on prophyrins in greening barley leaves

Seven-day-old dark-grown leaves were harvested and fed bipyridyl for 1 hr in the dark then for 15 hr in the light. The results (Table 2) show that bipyridyl is a powerful inhibitor of chlorophyll production; a 10 mM solution decreased chlorophyll by 83%. Protohaem was also lower, the maximum effect (47%) occurring at 5 mM. In parallel experiments similar results were obtained with *o*-phenanthroline, although this was a less effective inhibitor of porphyrin synthesis by *ca* one order of magnitude.

Effect on ALA formation from ^{14}C-precursors

Bipyridyl appears to promote the production of Pchlide from endogenous ALA in dark-treated barley leaves but to inhibit porphyrin synthesis in the light. It may be that bipyridyl-induced ALA synthesis does not occur in the light. To investigate this possibility glutamate-U-[^{14}C] or glycine-U-[^{14}C] (2.5 μCi) were fed to 2 cm segments from 7-day-old dark-grown leaves, for 4 hr in the light in the presence of bipyridyl and LA (an inhibitor of ALA condensation to porphobilinogen [8]). The effect of bipyridyl at 0.1 mM was to strongly inhibit ALA accummulation by 70% (Table 3). Higher concentrations of bipyridyl were less effective inhibitors of ALA formation (a feature noted in several related experiments). Bipyridyl, at 0.1 mM, strongly inhibited the incorporation of radioactive precursors into ALA (Table 4). However at 10 mM, bipyridyl increased the sp. act. of ALA-[^{14}C] from glutamate-[^{14}C] by 49%, against a decrease of 38% from glycine-[^{14}C], when compared to controls.

Protochlorophyllide regeneration and protochlorophyll formation in bipyridyl treated leaves

Eight-day-old dark-grown leaves were fed bipyridyl (10 mM) for 16 hr in the dark. Pchlide (P650) regeneration was measured *in vivo* after a 10 sec light flash. Mean regeneration rates in 3 experiments were 0.030A/100 min in bipyridyl treated leaves against 0.022A/100 min in controls. The slight effect of bipyridyl on regeneration rate is in contrast to the clear inhibition of ALA formation in the light (Table 3). In separate experiments, 8-day-old dark-grown leaves were fed ALA (10 mM) in the presence of bipyridyl (2.5 mM) for 16 hr in the dark. After a 3 min light flash, protohaem and non-photoconverted proto-

Table 4. Effect of bipyridyl on glycine-[^{14}C] and glutamate-[^{14}C] incorporation into δ-aminolaevulinic acid

^{14}C-Precursor and bipyridyl concentration (mM)	ALA Sp. act.	ALA Sp. act. as % controls
Glutamate-[^{14}C]		
10	101.4	149
1	32·1	47.1
0.1	12.0	17.6
0	68.2	
Glycine-[^{14}C]		
10	46.6	62.2
1	4.9	6.5
0.1	3.3	4.4
0	74.9	

Sp. act:ALA = DPM(ALA)/total DPM uptake × 10^{-4} × nmol ALA × g ft. wt. Seven-day-old dark-grown barley leaves were cut 4 cm below the tip and the top 2 cm discarded. The remaining 2 cm were fed glutamate-U-[^{14}C] (2.5 μCi, sp. act. 275 mCi/mM) or glycine-U-[^{14}C] (2.5 μCi, sp. act. 114 mCi/mM) in a solution of K–Pi buffer, pH 7.5, containing bipyridyl and LA (5×10^{-2} M). Feeding was for 4 hr in the light, after which ALA was extracted and purified before radioassay.

chlorophyll were extracted and determined. The results (Table 5) show that bipyridyl, in ALA-fed leaves, lowered protochlorophyll by 25% while increasing haem by only a small amount (11%). In the absence of exogenous ALA, bipyridyl increased protochlorophyll by 50%.

DISCUSSION

The effects of bipyridyl differ in dark and light-treated leaves; in the dark, bipyridyl induced an increase in photoconvertible Pchlide and a decrease in protohaem. However, there was no apparent stoichiometric relationship between the changes in the concentration of the two porphyrins. The turnover of the protohaem pool [9] in excised barley leaves of this age is *ca* 1.6 times slower than that of the protochlorophyllide pool [10], If bipyridyl caused a diversion of precursors from the haem pathway to Mg-prophyrin synthesis, then this diversion would not account for more than 20–30% of the increase in Pchlide. The evidence confirms that, in the dark, bipyridyl promotes the synthesis of ALA which, as in bean leaves [4], is utilised in the formation of Mg-porphyrins.

Table 5. Effect of bipyridyl on porphyrin production in δ-aminolaevulinic acid-fed leaves

Treatment	Protohaem nmol/g/fr. wt	Protohaem % control	Protochlorophyll (P630) nmol/g/fr. wt	Protochlorophyll (P630) % control
Bipyridyl + ALA	2.98	111	34.5	75
Bipyridyl	3.3	105	5.9	150
ALA	2.68	100	47.2	100
Control	3.15	100	3.9	100

Six-cm tops from 8-day-old dark-grown barley leaves were fed bipyridyl (2.5 mM, pH 7.5) with or without ALA (final concn 10 mM) for 16 hr in the dark. Protochlorophyll and protohaem were determined after a 3 min light treatment.

Bipyridyl-treated dark-grown bean leaves accumulate Mg-protoME from endogenous ALA [4]. Wheat leaves fed 8-hydroxyquinoline also accumulate Mg-protoME when supplied with exogenous ALA in the dark [5]. Barley leaves, however, do not accumulate Mg-protoME, except in relatively small amounts at high chelator concentrations. If one or more steps involved in Mg-protoME conversion to Pchlide requires a metal co-factor then, in barley, this metal does not appear to become limiting (at below 1 mM bipyridyl). Likewise there was no evidence that coproporphyrin accumulated in chelator-fed barley, which suggests that bipyridyl was unable to inactivate any metal-dependent coprogenase reaction; similar conclusions were reported for dark-grown bean leaves [4]. However, in dark-grown barley, in the presence of exogenous ALA, bipyridyl did inhibit protochlorophyll (P630) formation. It appears that when *substantial* quantities of ALA are being metabolized through the Mg-porphyrin pathway then a metal does become limiting. As bipyridyl did not significantly affect protohaem in the presence of ALA, it may be that a step after the formation of proto IX is inhibited in ALA-fed leaves, in the dark. In illuminated leaves, bipyridyl strongly inhibited chlorophyll synthesis again suggesting that when *substantial* quantities of ALA are being metabolised (as in greening leaves), bipyridyl induces one or more limiting steps. One of these steps, in the light, is ALA synthesis. Bipyridyl at high concentrations inhibited the incorporation of radioactivity from glycine-[^{14}C] into ALA, in the light. These findings suggest that there may be two pathways leading to ALA formation in barley leaves, one of which is inhibited by chelators such as 2,2'-bipyridyl and the other 'bipyridyl-stimulated'. If two ALA-forming enzymes are present, then the one utilizing glycine (ALA synthetase) is inhibited in the light and only at 10 mM bipyridyl is this inhibition partially overcome, perhaps after lowering a regulatory protohaem pool.

In higher animals and bacteria, ALA-synthetase activity is enhanced by bidentate chelators [11, 12]. If ALA-

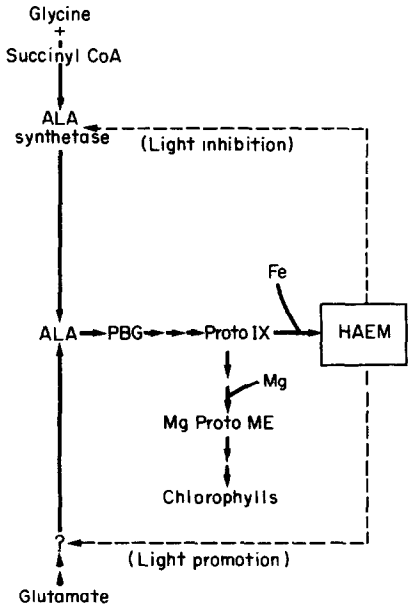

Fig. 2. The control of porphyrin synthesis in barley leaves.

synthetase is present in barley, then bipyridyl would enhance ALA formation. Such an enhancement appears to occur, but only in dark-treated leaves. ALA synthesis is inhibited by bipyridyl, in the light. This implies that an ALA-forming enzyme with characteristics similar to that of other organisms is operative in the dark in barley, but that, on illumination, a second enzyme system, differing from that of other organisms, becomes quantitatively more important. It is of interest that ALA-synthetase from potato-peel is reported to be inactivated by light [13].

Protohaem levels were lower in bipyridyl treatments in both light and dark-treated barley leaves probably owing, at least in part, to a depletion of iron as a substrate for the ferrochelatase reaction. Protohaem regulates ALA-synthetase activity in bacteria [14], mammals [15] and birds [16]. If protohaem regulation of ALA-synthetase occurs in barley, then a depletion of the protohaem pool(s), by bipyridyl, would enhance ALA-synthetase activity. This appears to be the case only in dark-grown leaves. Light increased the protohaem pool in barley [17] and decreased the rate of protohaem turnover [9]. It may be that, in the light, protohaem suppresses ALA-synthetase and promotes the formation of ALA from a glutamate derivative [2]. This proposal is outlined in Fig. 2.

EXPERIMENTAL

Barley seeds (*Hordeum vulgare*, cv Proctor) were soaked for 16 hr in H_2O before sowing in vermiculite. After germination at 25° in the dark for 6–10 days, the top 6 cm of the primary leaves were harvested. Chelator and ALA solns were prepared in 10 mM K–Pi buffer (pH 7.5), control treatments being buffer only

Quantitative assays. Chlorophyll, protochlorophyll(-ide) and protohaem were determined as before [17]. Mg-protoME was extracted in EtOAc–HOAc (4:1) after grinding leaf tissue in liquid N_2 [18]. The supernatant, after centrifugation (2500 *g*, 10 min), was partitioned against twice the vol. of 5 N HCl (× 2). The aq. phase was neutralised and then partitioned against EtOAc (× 2), this latter fraction being reduced in vol. (cold aspiration) before partitioning against equal vols of 0.1 N HCl (× 3) followed by 2.7 N HCl (× 3) to extract copro and proto IX respectively. The remaining EtOAc, after further vol. reduction, was partitioned against 5 N HCl (× 3). Porphyrins were estimated in HCl [7]. Pchlide regeneration was measured as described previously [19].

Radio-isotope experiments. Glutamate-U-[^{14}C] (275 mCi/mM) and glycine-U-[^{14}C] (114 mCi mM) were obtained from the Radiochemical Centre, Amersham, UK and purified by HV zone electrophoresis. Leaf segments (2 cm), fed isotope (2.5 μCi) for 4 hr in the light in the presence of bipyridyl and laevulinate (50 mM, pH 7.5), were washed vigorously in cold glutamate or glycine (1 mM), and then H_2O before extraction in hot (70°) 70% aq. EtOH. After partitioning against Et_2O, ALA was purified by electrophoresis [3] and estimated as the Ehrlich product [20]. Aliquots for counting were dried on to glass-fibre discs and radioactivity determined by liquid scintillation in toluene with PPO and POPOP.

Acknowledgements—AKS is grateful to The Royal Society for a grant for equipment. The authors would like to thank Mr. B. Perham for technical assistance.

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