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INHIBITION OF CHLOROPHYLL BIOSYNTHESIS BY α',α'-DIPYRIDYL DURING GREENING OF GROUNDNUT LEAVES

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Key Word Index—*Arachis hypogaea*; Leguminosae; groundnut; α',α'-dipyridyl; chlorophyllide; chlorophyll.

Abstract—The site of inhibition of chlorophyll biosynthesis by α',α'-dipyridyl was found to be at the level of conversion of chlorophyllide (672 nm) to chlorophyll (678 nm) during greening of groundnut leaves. This inhibition was partially reversed by certain divalent cations.

INTRODUCTION

Leaves of etiolated seedlings fail to synthesize chlorophyll in the dark but accumulate protochlorophyllide (Pchlide) with an A_{max} at 650 nm. On exposure to light this is converted to chlorophyllide which then undergoes enzymatic phytylation to yield chlorophyll [1]. The quantity of Pchlide accumulated in the dark can be considerably increased by the incubation of etiolated leaf material with δ-aminolevulinic acid (ALA)—an important intermediate of chlorophyll and heme biosynthesis [2]. However, the accumulated form has an A_{max} at 635 nm [3]. Addition of metal ion chelators such as α',α'-dipyridyl and 8-hydroxyquinoline to the etiolated leaves also enhances porphyrin synthesis and Pchlide accumulation [4–8]. In the light, however, the metal ion chelator dipyridyl totally inhibits chlorophyll synthesis [8]. It has been demonstrated that dipyridyl stimulates the synthesis of ALA but inhibits the conversion of magnesium protoporphyrin IX monomethyl ester to Pchlide in the dark [7]. The reason for the inhibition of chlorophyll synthesis from Pchlide by dipyridyl in light is not known.

In the present communication, we confirm some of the earlier work on dipyridyl and further provide data which suggest that it inhibits the conversion of chlorophyllide to chlorophyll in light. This inhibition is partially reversed by certain divalent cations.

RESULTS AND DISCUSSION

The studies on the effect of dipyridyl on the accumulation of chlorophyll in greening leaves showed that at 0.5 mM dipyridyl concentration chlorophyll synthesis was completely blocked. The dipyridyl also inhibited the synthesis of ALA in the presence of levulinic acid in light (data not shown). However, in etiolated leaves the synthesis of Pchlide was stimulated two-fold by 0.5 mM or 1 mM dipyridyl concentration. Also in the dark the ALA accumulation was stimulated three-fold by 1 mM dipyridyl in the presence of levulinic acid (data not shown). The extent of stimulation and inhibition of the synthesis of chlorophyll and its precursors by dipyridyl under different conditions, as mentioned above, is very similar to that observed by Hendry and Stobart [8] recently. The earlier studies have shown the probable steps of the chlorophyll biosynthesis pathway affected by dipyridyl in the dark. We have, therefore, focused our attention on its inhibitory effect on chlorophyll synthesis in light by using spectrophotometry and TLC.

Figure 1 shows the *in vivo* absorption spectra of etiolated leaf under different incubation conditions. The leaf incubated in the dark with either ALA or dipyridyl or a mixture of the two on exposure to light for 2 min shows an A_{max} at 672 nm which is similar to that observed for an untreated leaf exposed to light for 2 min. However, continued exposure to light for 3 hr of the ALA- or dipyridyl-treated material does not result in the shift of the peak to 678 nm. Even the 18 hr exposure to light of the leaf treated with dipyridyl still shows an A_{max} at 672 nm. The untreated leaf under identical conditions shows an A_{max} at 678 nm characteristic of chlorophyll accumulation. These data demonstrate that the ALA or dipyridyl treatment prevents conversion of 672 nm form to 678 nm form although the treatment does not prevent accumulation of 672 nm form from Pchlide absorbing at 635 nm. The accumulated chlorophyllide with A_{max} 672 nm in the presence of dipyridyl was extracted and examined by TLC. It moved with an R_f value of 0.06 as against an R_f value of 0.96 for chlorophyll in the solvent system used. TLC analysis showed that the 672 nm chlorophyllide had an orange-red fluorescence. This spot was eluted and its excitation and emission characteristics were studied. Figure 2 shows that it has an emission maximum at 667 nm (uncorr.) in acetone when excited with 390 nm or 440 nm. The emission characteristics are in agreement with the conclusion that 672 nm peak is chlorophyllide [9]. It thus appears that dipyridyl does not allow synthesis of chlorophyll from chlorophyllide. In view of the fact that information is already available on the phototransformation of Pchlide accumulated in the presence of exogenously added ALA we have studied the phototransformation of Pchlide in the presence of dipyridyl. It may be mentioned here that ALA- and dipyridyl-treated leaves on incubation in the dark accumulate 635 nm Pchlide; however, if the etiolated leaf which has accumulated photoconvertible 650 nm Pchlide is transferred to light in the presence of ALA, chlorophyll synthesis is not blocked. The dipyridyl does not permit even the photoconversion of the 650 nm Pchlide form into chlorophyll (Fig. 1).

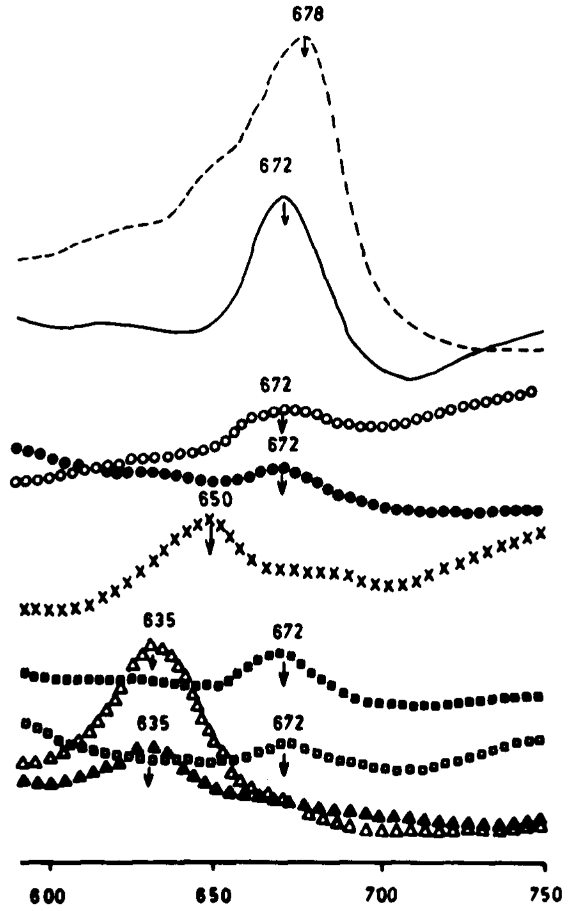

Fig. 1. *In vivo* absorption spectra of 12-day-old etiolated groundnut leaf under different conditions. ×, Etiolated leaf; ----, leaf exposed to light (800 lx) for 18 hr; ———, leaf exposed to light for 18 hr in the presence of 1 mM dipyridyl; ○, leaf exposed to light for 2 min; ●, leaf incubated in the dark with 10 mM ALA and 1 mM dipyridyl for 18 hr and then exposed to light for 2 min; △, leaf incubated in the dark for 18 hr with 10 mM ALA; ▲, leaf incubated in the dark for 18 hr with 1 mM dipyridyl; ■, leaf incubated in the dark for 18 hr with 10 mM ALA and then exposed to light for 3 hr; □, leaf incubated in the dark for 18 hr with 1 mM dipyridyl and then exposed to light for 3 hr.

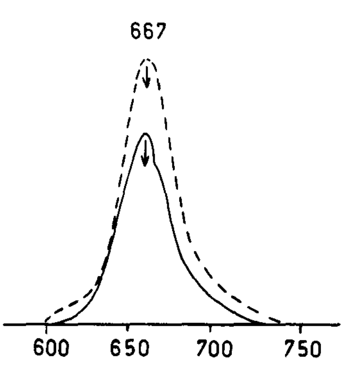

Fig. 2. Fluorescence emission spectra (uncorrected) of chlorophyllide eluted in 100 % acetone from the plates after TLC. ----, Excitation at 440 nm; ——— excitation at 390 nm.

The prevention of phototransformations of the 635 nm form accumulated in the dark in the presence of dipyridyl suggests either that this 635 nm form cannot be converted to the 678 nm form irrespective of the presence or absence of dipyridyl, or that dipyridyl blocks the conversion of 672 nm form to 678 nm form. It is known that the 635 nm form accumulated in the dark in the presence of exogenously supplied ALA can be converted into the 678 nm form [10] by the use of a short duration flash followed by a 3 min dark period. Also it is known that 635 nm Pchlide accumulated in ALA-treated samples can be slowly transformed into 650 nm photoconvertible protochlorophyllide [11, 12]. It thus appears that conversion of 635 nm Pchlide to normal chlorophyll is possible under certain conditions. It should, however, be noted that under continuous illumination the 635 nm form is not phototransformed into chlorophyll. It thus appears that one of the reasons for the blocking of chlorophyll synthesis in light by dipyridyl could be the production of 635 nm Pchlide. However, the observation that even the 650 nm photoconvertible form accumulated in etiolated leaf cannot be transformed into chlorophyll in the presence of dipyridyl (Fig. 1) suggests an additional step located beyond phototransformation of 650 nm Pchlide which is sensitive to dipyridyl. We have considered the following two possibilities. (a) That dipyridyl blocks the conversion of the 672 nm form to the 678 nm form and, (b) that the 672 nm form produced in the presence of dipyridyl in light from the 650 nm form may not be identical to that produced in untreated leaf which appears after a 684 nm form. This possibility had to be considered because it was previously shown that a 672 nm inactive form is produced directly from P650 (see [13]).

In order to understand which of the two above-mentioned possibilities is responsible for the dipyridyl effect, the following experiments were conducted. The leaves that had accumulated 650 nm protochlorophyllide were exposed to a short period of illumination (30 sec). The conversion of 650 nm form to 684 nm form was detected (see Fig. 3, curve a) and then they were divided into two sets. One set was exposed to light without addition of dipyridyl and the other exposed to light in the presence of 1 mM dipyridyl. The *in vivo* absorption spectra indicated that in the untreated material the 678 nm form was produced (Fig. 3, curve c) whereas in the dipyridyl treated set the 672 nm form was accumulated (Fig. 3, curve b). This experiment indicated that dipyridyl did not interfere with the transformation of the 684 nm form to the 672 nm form but it did not allow conversion of the 672 nm form into the 678 nm form. This was further confirmed by another experiment in which the untreated etiolated leaves were first exposed to light to convert the 650 nm form into the 672 nm form (Fig. 4, curve a). The leaves were then divided into two lots. One lot was exposed to light in the absence of dipyridyl and another was exposed to light in the presence of 1 mM dipyridyl. As expected, the dipyridyl-treated leaves did not allow conversion of the 672 nm form to the 678 nm form (Fig. 4, curve b) although in untreated leaf the 678 nm peak indicating chlorophyll accumulation was seen. These data show that in light, dipyridyl blocks the conversion of the 672 nm form to the 678 nm form (Fig. 4, curve c).

It has been shown [14] that the conversion of the 672 nm to the 678 nm form is associated with phytylation and that this is mediated by the enzyme chlorophyllase [15]. In view of the fact that dipyridyl is a metal ion chelator it may block the activity of chlorophyllase if this enzyme requires metal ions for its activity. If so, one would expect that addition of appropriate cations should reverse the effect of dipyridyl. Experiments to test this possibility were carried out. The results showed that of the different divalent cations only Zn^{2+}, Co^{2+} and Fe^{2+} were able partially to reverse the inhibitory effect of dipyridyl. The data presented in Table 1 show that at 1 mM Zn^{2+} the reversal was as high as *ca* 40%. Cobalt at 1 mM concentration was able to bring about 20% while Fe^{2+} at the same concentration gave *ca* 17% reversal. The fact that in this experiment chlorophyll was detected in the Zn^{2+}, Co^{2+} and Fe^{2+}-treated leaves even in the presence of dipyridyl indicates that the conversion of the 672 nm form to the 678 nm form is taking place. It thus appears that Zn^{2+}, Co^{2+} and Fe^{2+} can to some extent reverse the inhibition imposed by dipyridyl on chlorophyll synthesis in light. We do not think that the reversal is due primarily to the effect of these metal ions prior to protochlorophyllide formation since it has previously been shown that such metal ions, if anything, inhibit the synthesis of porphyrins induced by dipyridyl [7].

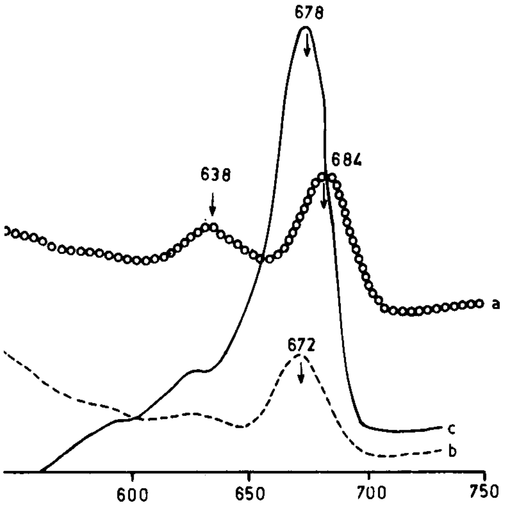

Fig. 3. The effect of dipyridyl on transformation of accumulated chlorophyllide. (a) *In vivo* absorption spectra of etiolated leaf exposed to light for 0.5 min. (b) *In vivo* absorption spectra of the leaf treated as in (a) but after continued exposure in light for 3 hr in the presence of 1 mM dipyridyl. (c) *In vivo* absorption spectra of the leaf treated as in (a) but after continued exposure in light for 3 hr.

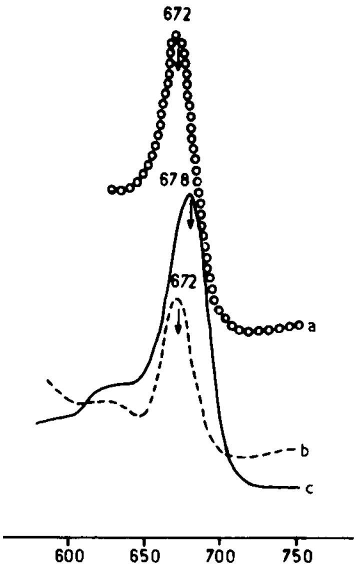

Fig. 4. The effect of dipyridyl on transformation of chlorophyllide (672 nm). (a) *In vivo* absorption spectra of etiolated leaf exposed to light for 3 min. (b) *In vivo* absorption spectra of the leaf treated as in (a) but after continued exposure in light for 3 hr in the presence of 1 mM dipyridyl. (c) *In vivo* absorption spectra of the leaf treated as in (a) but after continued exposure in light for 3 hr.

EXPERIMENTAL

The experimental procedure as described in ref. [7] was followed. Seeds of *Arachis hypogaea* were germinated in moistened vermiculite in complete darkness at 25°. All manipulations of plant material were done under a dim green safelight [16]. 12-Day-old etiolated leaves were vacuum

Table 1. Effect of various metal ions on the dipyridyl-induced inhibition of chlorophyll synthesis

First 6 hr dipyridyl	Additional 18 hr	Chlorophyll (μg/g fr. wt)	% Reversion
−	H_2O	316	—
+	H_2O	—	—
+	Dipyridyl	—	—
+	Zn^{2+} (5 mM)	30.1	9.5
+	Zn^{2+} (2 mM)	46.2	14.6
+	Zn^{2+} (1 mM)	125.6	39.7
+	Zn^{2+} (0.5 mM)	88	27.8
+	Co^{2+} (1 mM)	70.6	22.2
+	Fe^{2+} (1 mM)	54.8	17.3

The leaves of 12-day-old etiolated groundnut seedlings were treated as described in the Experimental, incubated in light for 6 hr and then transferred to water, 1 mM dipyridyl or the various metal chloride solutions.

infiltrated with either 1 mM α',α'-dipyridyl or H_2O containing 1% sucrose plus 0.1 mM HCl. The leaves were then transferred to Petri dishes (5 cm) containing filter paper moistened with either of these solns and incubated under fluorescent light (800 lx) for the required period of time. Those leaves requiring an additional incubation were first rinsed with H_2O to remove excess chelator and then incubated with the appropriate soln in light. The absorption spectrum of a single leaf was recorded with a recording spectrophotometer. The leaf was kept in a special holder and placed in the sample compartment of the spectrophotometer. The reference cells contained a single thickness of Whatman No. 1 filter paper. Recording was done in the 400–700 nm region. The fluorescence emission spectra were studied using a spectrophoto-fluorometer coupled to an x–y recorder.

Chlorophylls were estimated by the method of ref. [17].

Extraction and identification of Pchlide, chlorophyllide and chlorophyll *in vitro* were done by TLC using the procedures of ref. [18]. The samples were spotted on Si gel H plates (activated at 100° for 1 hr) and developed with C_6H_6–EtOAc–EtOH (4:1:1). The front was allowed to move *ca* 15 cm from the origin. The plate was dried under a stream of N_2 and then examined under long-wavelength UV light to detect different fluorescing areas. These areas were eluted in Me_2CO and fluorescence emission spectra were recorded at room temp.

Chemicals were from Sigma and reagents were analytical grade.

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