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The Development of Plastocyanin in Greening Bean Leaves

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The plastocyanin content of etiolated bean leaves (*Phaseolus vulgaris* L.) was measured, and the development of the protein in response to light was followed. Measurements were made by quantitative extraction of plastocyanin and a sensitive assay with an O_2 electrode. The electron-paramagnetic-resonance (e.p.r.) signal of oxidized plastocyanin was used as an independent check on the validity of the assay method, and on the thoroughness of extraction. After an initial lag period, the amount of plastocyanin in greening bean leaves increased to reach a maximum after 50h illumination. The chlorophyll/plastocyanin ratio reached a maximum value of 200 irrespective of the light intensity at which greening was carried out, suggesting that the synthesis of the two components is co-ordinated. Experiments involving treatment of etiolated seedlings with brief periods of light of different spectral composition indicated that phytochrome is involved in plastocyanin synthesis. The lack of inhibition of plastocyanin synthesis by specific inhibitors of chloroplast protein synthesis suggests that the protein is synthesized on cytoplasmic ribosomes. The data are discussed in relation to the development of ferredoxin in greening bean leaves.

The study of chloroplast development during the greening of etiolated leaves has attracted considerable interest during recent years (Kirk & Tilney-Basset, 1967). However, comparatively little is known about the development of photosynthetic electron-transport components owing to the difficulty of quantitative extraction and assay of such compounds.

In a previous publication (Haslett *et al.*, 1973), we presented results of a quantitative study on the development of ferredoxin, the soluble iron–sulphur protein involved in photosynthetic electron transport at Photosystem I. The present paper concerns the results of a similar study on the development of the copper protein, plastocyanin (Katoh, 1960). This protein has a redox potential of +370mV (Katoh *et al.*, 1962), and most plastocyanins have been found to have a molecular weight of approx. 10500 and to contain one atom of copper per molecule (Ramshaw *et al.*, 1973). Plastocyanin acts as an electron-transfer protein at a site close to Photosystem I in chloroplasts (Katoh & Takamiya, 1963).

In leaves of etiolated barley, Plesnicar & Bendall (1970, 1972, 1973) reported the presence of plastocyanin and found that this did not increase significantly over a period of 24h illumination, when the values were expressed per unit fresh weight.

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Experimental

Greening experiments

Plant material used in these experiments was *Phaseolus vulgaris* L., var. Canadian Wonder. Conditions for cultivation and greening have been described previously (Haslett *et al.*, 1973).

Extraction of plastocyanin

All extraction procedures were carried out at 4°C. Leaves (5–10g) were homogenized with a pestle and mortar, in 25ml of medium containing 20mM-Tris–HCl–buffer (pH7.4) and 0.035M-NaCl. Sand was added to facilitate grinding, and 1g of insoluble polyvinylpyrrolidone was added to adsorb polyphenols. After squeezing through two layers of 40-mesh nylon, filtrates were sonicated with a Dawe 'Soniprobe' model 1130/A (Dawe Instruments, London W.3, U.K.). This was used at a current of 4.5A. During sonication, the temperature of the filtrate was kept below 10°C by periodic immersion of the sonication vessel in liquid N_2. Maximum release of plastocyanin from green leaves was observed after 1min sonication under these conditions (see Fig. 1). The volume of filtrate during sonication (10–100ml) or the concentration of leaves per filtrate (5–25 leaves per 50ml) did not affect the release of plastocyanin by sonication.

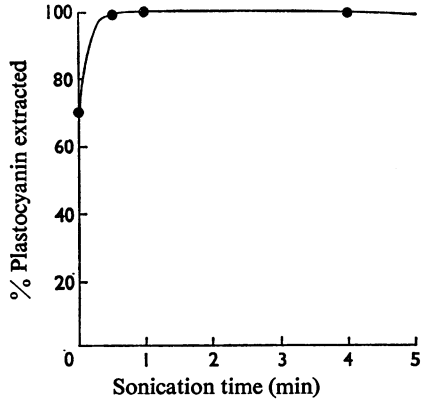

Fig. 1. *Effect of the duration of sonication on the release of plastocyanin from greenhouse-grown bean leaves*

Conditions are given in the text.

Table 1. *Release of plastocyanin on re-extraction of leaf tissue*

The experiment was carried out on greenhouse-grown bean leaves. Methods of plastocyanin extraction and assay are described in the text.

Source of extraction	Plastocyanin released (μg/extract)	Percentage of plastocyanin extracted
1. Leaf tissue	320.0	87.0
2. Leaf debris from step 1	30.0	9.4
3. Pellet from step 1	8.0	2.2

Sonicates were centrifuged at 140000*g* for 30min and portions of the supernatants were taken for assay. In order to test the efficiency of the extraction technique, both the leaf debris after homogenization and the pellet obtained after centrifugation of the sonicated filtrate were resuspended in a small volume of extraction medium and resonicated for 1min. Assay of supernatants after the centrifugation of such samples (see Table 1) revealed that approx. 90% of the total plastocyanin was removed from the leaf during the initial extraction, and 10% remained in the leaf debris. In greening experiments, leaves were extracted once only and the values for plastocyanin content were increased by 10% to allow for this loss.

Another approach used to investigate the efficiency of plastocyanin extraction involved measurement of the e.p.r. (electron paramagnetic resonance) signal of oxidized plastocyanin *in situ* in samples prepared from the pellets obtained after centrifugation of filtrates. Oxidized plastocyanin in chloroplasts has been shown to give rise to an e.p.r. spectrum similar to that of the purified protein (Malkin & Bearden,

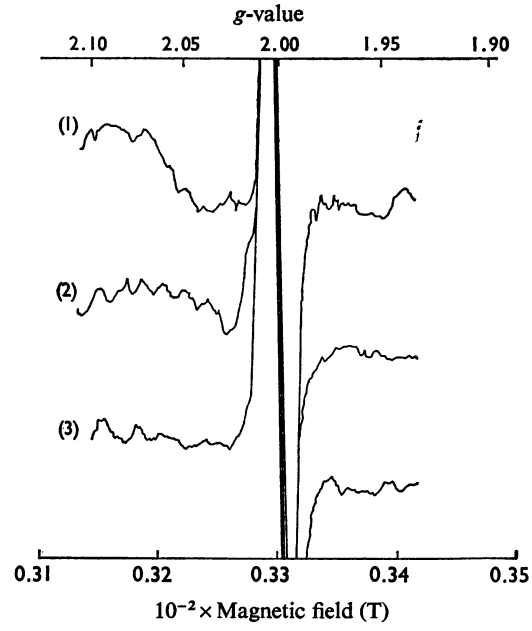

Fig. 2. *E.p.r. spectra of bean-leaf fragments after extraction of plastocyanin*

(1) Fragments from homogenization of leaves with MSE Atomix apparatus. (2) Fragments from homogenization with pestle and mortar. (3) Fragments from homogenization with pestle and mortar plus subsequent sonication. Instrument settings were: temperature, 77°K; microwave frequency, 9.17GHz; modulation amplitude, 10G. The signal of oxidized plastocyanin is represented by the dip in the e.p.r. spectrum around $g = 2.05$ (cf. Fig. 3). The ordinate is the first derivative of the microwave absorption in arbitrary units $\left(\frac{d\chi''}{dH}\right)$.

1973). Excess of potassium ferricyanide was added to each e.p.r. sample (volume 100μl) to ensure full oxidation of plastocyanin. Such samples were centrifuged at 500*g* for 5min and the supernatants decanted in order to sediment membrane fragments, thereby increasing the sensitivity of e.p.r. measurements. Samples for e.p.r. were placed in darkness for 1h before freezing to decrease the size of the e.p.r. signal attributable to the oxidized pigment P-700 (Bearden & Malkin, 1972). The results of such experiments (shown in Fig. 2) must be regarded as qualitative, since the chlorophyll content varied between samples. However, they indicate that pestle-and-mortar grinding followed by sonication effectively eradicated the e.p.r. signal indicative of plastocyanin. Malkin & Bearden (1973) have reported similar results after sonication of spinach chloroplast preparations.

Bioassay of plastocyanin

The method was based on that of Hauska *et al.*

(1971). It involved measurement of the rate of electron transport through Photosystem I in chloroplast fragments. This rate was measured as O_2 uptake during autoxidation of the ultimate electron acceptor, Methyl Viologen. Measurements were made with a Clark-type O_2 electrode (Rank Bros., Bottisham, Cambs., U.K.). The reaction mixture contained the following: Tris–HCl buffer (pH 8.5), 150 μmol; 3-(3,4-dichlorophenyl)-1,1-dimethylurea, 1 μmol; Methyl Viologen, 5 μmol; sodium ascorbate, 50 μmol; digitonin, 0.017% (w/v); NaN_3, 30 μmol; P_1S_1 spinach chloroplasts (Whatley & Arnon, 1963), 0.05 mg of chlorophyll. After the addition of 0.1–0.3 ml of leaf extract, the volume of the reaction mixture was made up to 3.0 ml with distilled water.

A standard curve was made, by using spinach plastocyanin purified by the method of Plesnicar & Bendall (1970), for each set of samples assayed. Some O_2 uptake was detected in the absence of illumination and this was subsequently subtracted from the light-dependent rate. Plastocyanin from spinach and French bean gave identical rates of O_2 uptake per mol of plastocyanin. With this assay system, amounts of plastocyanin ranging from 1.0 to 4.0 nmol could be measured with a variation of ±5%.

Assay of plastocyanin by e.p.r. spectroscopy

This method was primarily developed as an independent check on the bioassay method. Because of the relatively low sensitivity of this method, extracts were concentrated 10–20-fold with a Diaflo diafiltration unit containing a PM 10 membrane (Amicon Ltd., High Wycombe, Bucks., U.K.).

The method was similar to that used in the assay of ferredoxin (Haslett *et al.*, 1973). Since only the

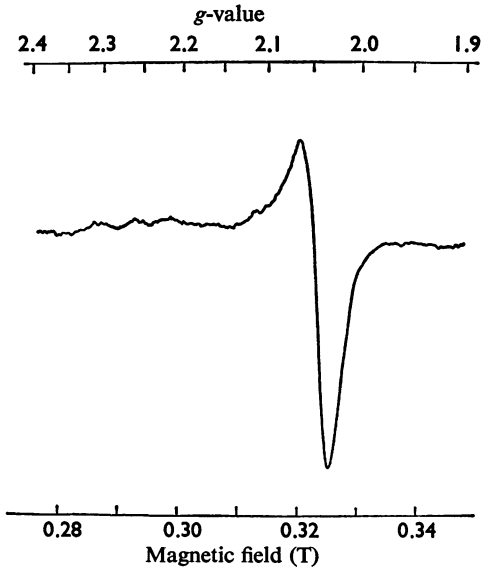

Fig. 3. *E.p.r. spectrum of oxidized purified bean plastocyanin*

Instrument settings were similar to those of Fig. 2, but at a lower gain setting.

Table 2. *Comparison of bioassay and e.p.r. assay for plastocyanin*

Samples assayed in all three experiments were prepared from 14-day-old greenhouse-grown bean leaves. Each extract was concentrated 20-fold and assayed by two methods as described in the text.

	Plastocyanin content of extract (μg/ml)	
Expt. no.	E.p.r. assay	Bioassay
1	71.3	66.5
2	68.0	66.8
3	45.0	47.0

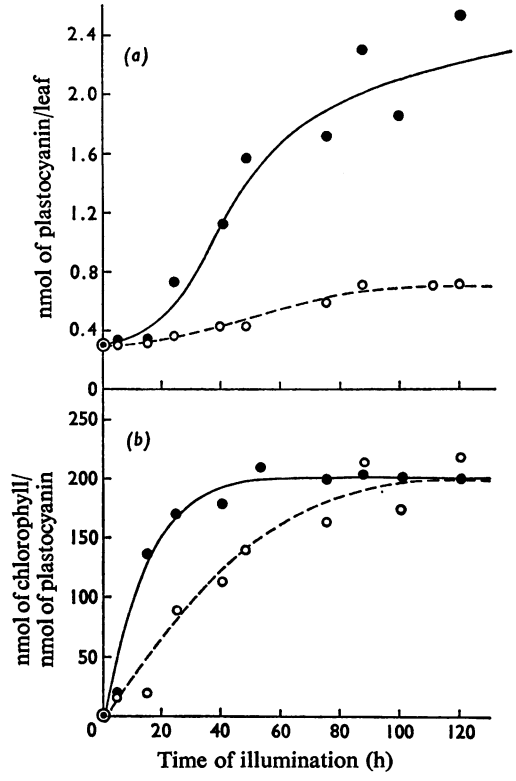

Fig. 4. *Development of (a) plastocyanin and (b) the chlorophyll/plastocyanin ratio during continuous illumination at 2500 lx (●) and 60 lx (○)*

For details see the text.

Table 3. *Effect of short light treatment on synthesis of plastocyanin and chlorophyll*

Light intensities and spectral characteristics of the illumination system used are described by Bradbeer (1971).

Treatment	Plastocyanin content per leaf (nmol)	Chlorophyll content per leaf (nmol)
Before illumination	0.237	0
No illumination	0.221	0
Red (5 min)	0.385*	3.70
Red/far-red (5 min/20 min)	0.229†	3.15
Far-red (20 min)	0.238	2.06
Blue (20 min)	0.239	3.02
White (5 min)	0.476	3.94

* Significantly different from 'No illumination' at $P = 0.01$ as determined by Student's t test.

† Significantly different from 'Red' at $P = 0.01$ as determined by Student's t test.

Table 4. *Effect of inhibitors of chloroplast translation on development of plastocyanin and chlorophyll*

Seedlings were treated as described previously (Haslett *et al.*, 1973). D- and L-*threo*-chloramphenicol were used at a concentration of 1 mg/ml and lincomycin at a concentration of 0.05 mg/ml. After the period of inhibitor uptake (6h), average concentrations per leaf were as follows: D- and L-*threo*-chloramphenicol, 200 μg; lincomycin, 9.0 μg. Seedlings were greened for 50h at 2500 lx and 25°C.

Treatment	Plastocyanin content per leaf (nmol)	Chlorophyll content per leaf (nmol)
Before illumination	0.26	0
Water	1.50	202.0
L-*threo*-Chloramphenicol	1.56	99.0
D-*threo*-Chloramphenicol	1.58	40.6*
Lincomycin	1.51	148.6

* Significantly different from L-*threo*-chloramphenicol-treated sample at $P = 0.05$ as determined by Student's t test.

oxidized form of plastocyanin shows an e.p.r. signal, excess of potassium ferricyanide was added to the samples. The plastocyanin concentration was taken as being proportional to the size of the e.p.r. signal at $g = 2.05$ (see Fig. 3). A standard curve of peak height versus plastocyanin concentration was drawn by using purified spinach or bean plastocyanin. Samples containing as little as 2.0 nmol of plastocyanin could be assayed by this method. Table 2 shows that the two independent assay methods gave close agreement on the plastocyanin content of a single leaf extract, indicating the absence of substances interfering with the rate of electron transport in the chloroplast bioassay system. In all experiments relating to the development of plastocyanin, only the chloroplast bioassay system was used.

Chlorophyll measurement

The chlorophyll content of leaves was measured by the method of Arnon (1949).

Results

Plastocyanin development during continuous illumination

From ten independent measurements, etiolated leaves from 14-day-old bean seedlings were found to contain 0.262 ± 0.03 (S.D.) nmol per leaf. Measurements on a single batch of leaves, i.e. from seedlings grown at the same time, showed a standard deviation of 4%, indicating that the major source of error was in physiological variation between different batches of plants.

When such seedlings were illuminated at a light-intensity of 2500 lx an initial lag period of approx. 5h occurred, during which little or no net synthesis of plastocyanin was observed on the basis of weight per leaf (see Fig. 4*a*). The rate of plastocyanin synthesis subsequently increased, reaching a maximum after 25h illumination. The chlorophyll/plastocyanin ratio increased rapidly from the onset of illumination, reaching a constant maximum value of 195 ± 13 (S.D.) after 30h illumination (Fig. 4*b*).

Table 5. *Comparison of plastocyanin, ferredoxin and chlorophyll development*

Data relating to the development of ferredoxin are taken from Haslett *et al.* (1973).

	Plastocyanin	Ferredoxin	Chlorophyll
Presence of lag	Present	Absent	Present
Content of etiolated leaf (nmol)	0.262 ± 0.03	0.104 ± 0.31	0
Content of fully greened leaf (nmol)	2.5	1.35	500
Chlorophyll/component ratio in fully greened leaves	195 ± 13	356 ± 40	—
Site of translation leading to synthesis	Cytoplasm	Cytoplasm	Cytoplasm/chloroplast
Type of light controlling synthesis	Red/far-red	Red	Red+red/far-red

When greening was performed at a light-intensity of 60lx, the pattern of plastocyanin development was similar to that seen at 2500lx. However, the length of the lag phase was increased and the maximum rate of plastocyanin synthesis was lower. In addition, the maximum amount of plastocyanin found in leaves greened under such conditions was greatly decreased. At 60lx the chlorophyll/plastocyanin ratio also increased in a manner similar to that seen at 2500lx. However, the rate of increase was less, and the maximum value was reached after a longer period of greening. The final chlorophyll/plastocyanin ratio reached under such conditions was measured as 199 ± 27 (S.D.), which was not significantly different from the value obtained during greening at 2500lx.

Effect of light composition on plastocyanin development

The 14-day-old etiolated bean seedlings were exposed to short periods of illumination with light of different spectral composition. The illumination system used was identical with that described by Bradbeer (1971). Seedlings were illuminated for brief periods at intervals of 24h for 3 days and otherwise kept in the dark. At the end of this period, plastocyanin was extracted from the leaves and assayed.

The results of such experiments are summarized in Table 3. Plastocyanin synthesis was stimulated by red light, and this stimulation was reversed by subsequent illumination by far-red light.

Site of plastocyanin synthesis

The effects of lincomycin (Ellis & Hartley, 1971) and D-*threo*-chloramphenicol (Rendi & Ochoa, 1962; Ellis, 1969) on the synthesis of plastocyanin are shown in Table 4. Both compounds interact with the 70S ribosomes of chloroplasts but not the 80S ribosomes of the cytoplasm, thus specifically inhibiting protein synthesis in chloroplasts at the level of translation. In experiments involving D-*threo*-chloramphenicol, the stereoisomer L-*threo*-chloramphenicol was used as a control, in order to measure any non-specific effects of the D-isomer on cytoplasmic protein synthesis (Ireland & Bradbeer, 1971).

Table 4 shows that plastocyanin synthesis is not significantly affected by inhibitors of chloroplast translation, indicating that such synthesis does not require translational steps on chloroplast ribosomes. Its synthesis must therefore be cytoplasmic.

Discussion

Previous studies on barley leaves (Plesnicar & Bendall, 1972, 1973) have shown that the plastocyanin concentration in greening leaves remains constant, if expressed on a fresh-weight basis. However, the leaves are expanded during this process with consequent increase in fresh weight. This means that synthesis of plastocyanin is taking place. For this reason we have expressed the plastocyanin content in terms of weight per leaf, and compared its synthesis with that of chlorophyll.

The characteristics of development of plastocyanin in greening bean leaves appear to be different in several respects from those of ferredoxin (Haslett *et al.*, 1973) (Table 5), although both are soluble electron-transfer proteins and both appear to be synthesized on cytoplasmic ribosomes. First, the synthesis of plastocyanin induced by red light is inhibited by far-red light, indicating that it is controlled by phytochrome. On the other hand the synthesis of ferredoxin is stimulated by red light and such stimulation is unaffected by subsequent far-red illumination (Haslett *et al.*, 1973). Secondly, ferredoxin synthesis shows no lag at the onset of illumination of etiolated leaves, whereas plastocyanin, like chlorophyll and several other photosynthetic components (e.g. Gregory & Bradbeer, 1973), shows a lag phase before synthesis proceeds at a maximal rate. Thirdly, the ratio of chlorophyll to plastocyanin becomes constant at an earlier stage than the ratio of chlorophyll to ferredoxin. Finally, the synthesis of ferredoxin is stimulated by inhibitors of chloroplast protein synthesis such as D-*threo*-chloramphenicol or lincomycin, whereas that of plastocyanin is not significantly affected. These differences suggest that the synthesis of the two proteins may be controlled in different ways.

In spite of these differences, the synthesis of plastocyanin and ferredoxin results in a constant molar proportion of approx. 400 chlorophyll: 2 plastocyanin:1.1 ferredoxin, over a wide range of light-intensity, although the rates of synthesis and the final concentrations reached are considerably lower at lower light-intensity. It seems therefore that the synthesis of chlorophyll, plastocyanin and ferredoxin in greening bean leaves is in some way co-ordinated.

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