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Quantitative Studies on Ferredoxin in Greening Bean Leaves

By B. G. HASLETT* and R. CAMMACK
Department of Botany, King's College (University of London), 68 Half Moon Lane, London SE24 9JF, U.K.
and F. R. WHATLEY
Department of Botany, University of Oxford, South Parks Road, Oxford OX1 3RA, U.K.

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Two methods of measuring small amounts of the iron–sulphur protein ferredoxin are described. One involves measurements of the signal at $g = 1.96$ produced by reduced ferredoxin in an e.p.r. (electron-paramagnetic-resonance) spectrometer; the other depends on the rate of ferredoxin-dependent electron transport in a chloroplast bioassay measured in an O_2 electrode. These methods of measurement were used to examine the development of ferredoxin during the greening of etiolated bean leaves. Ferredoxin is present in low concentrations in the leaves and cotyledons of 14-day-old etiolated beans (*Phaseolus vulgaris* L. var. Canadian Wonder), and develops in a linear manner with time when the leaves are illuminated. This synthesis appears to be independent of chlorophyll synthesis during the early stages of greening. However, the chlorophyll/ferredoxin ratio reaches a final value of approx. 360 irrespective of the light intensity, indicating the existence of a control mechanism operative in deciding the stoicheiometry of these components in the mature chloroplast. The ferredoxin synthesis appears to be light-dependent, and red light is the most effective in its promotion. The effect of red illumination is not reversed by far-red light, indicating the absence of a phytochrome control of ferredoxin synthesis. From experiments using specific inhibitors of chloroplast protein synthesis, it is concluded that ferredoxin is synthesized on cytoplasmic ribosomes.

Beans grown in the dark contain no chlorophyll and only very rudimentary plastids called etioplasts, which contain small amounts of some components of photosynthesis (Kirk & Tilney-Bassett, 1967).

During continuous illumination of such plants, a number of structural and biochemical changes take place, leading to the formation of chloroplasts. The structural changes consist of a breakdown of the paracrystalline prolamellar body and the formation of lamellae, with the eventual formation of grana. Simultaneously, characteristic changes occur in the activities of many of the photosynthetic carbon-cycle enzymes after the onset of continuous illumination (Bradbeer, 1969). These activity changes appear to be due to protein synthesis *de novo*, some enzymes being synthesized on cytoplasmic ribosomes whereas others are formed inside the chloroplast (Ireland & Bradbeer, 1971).

In recent years, studies on the development of photosynthetic electron-transport processes in chloroplasts have mainly concerned the partial reactions of the electron-transport chain with artificial electron donors and acceptors. Such research has largely been directed toward finding the earliest stage at which the two photosystems become functional (see, e.g., Gyldenholm & Whatley, 1968). Comparatively little information is available on the development of individual components of the electron-transport chain, probably owing to the difficulty of quantitative extraction and assay of the components concerned.

Melandri *et al.* (1967) and Phung Nhu Hung *et al.* (1970) have studied the development of ferredoxin during greening of etiolated bean and barley respectively. Both groups detected ferredoxin in etiolated leaves, and suggested that in the early stages of greening there was no correlation between the biosynthesis of chlorophyll and of ferredoxin. This was the view of Hudock & Levine (1964), who investigated the changes in several chloroplast components of *Chlamydomonas reinhardii* (strain Y-2) during light and dark periods.

The present paper gives a more-detailed account of the development of ferredoxin during greening of etiolated bean leaves, and attempts to show how this relates to the development of chlorophyll. Previous work has been expressed in terms of activity changes, whereas the present data are given as mol of ferredoxin per leaf. This allows a calculation of the molar ratios of chlorophyll to ferredoxin.

The method of ferredoxin determination used in the present work was developed from the method of San Pietro (1963). The latter method

* Present address: Department of Botany, University of Durham, Durham DH1 3LE, U.K.

involved measurement of ferredoxin-dependent NADP photoreduction in broken spinach chloroplasts by means of optical absorption increase at 340nm. In the present paper a similar system is used, but the rate of ferredoxin-dependent electron transport is determined from the O_2 release at photosystem II, as measured by an O_2 electrode. This method avoids the inaccuracies due to non-specific absorption changes at 340nm and light-scattering by the chloroplasts. Such difficulties might be overcome by more elaborate spectrophotometric methods, such as dual-wavelength spectrophotometry, but the advantage of the O_2-electrode method is its relative cheapness and simplicity.

In addition, we have used a method of measurement based on the size of the e.p.r. (electron-paramagnetic-resonance) signal centred around $g = 1.96$ produced by reduced ferredoxin. This method was used as a check on the specificity of the bioassay, although it is less sensitive than the latter. The method is quick and convenient, and is the most-sensitive physical method so far devised. By contrast, measurements of optical absorption of ferredoxin at 420nm (Whatley *et al.*, 1963) are relatively insensitive and are only suitable for concentrated and purified ferredoxin.

Experimental

Greening experiments

Plant material used in these experiments was *Phaseolus vulgaris* L., var. Canadian Wonder. These plants were grown on vermiculite in darkness at 23°C for 14 days, before being used in greening experiments. Plants were illuminated in a Controlled Environment growth chamber, the light source being fluorescent tubes (Lifeline Sylvana) of an intensity indicated in the individual experiments. At the beginning of each greening experiment, plants were cut at the hypocotyl and placed together in water to average out any physiological differences between pots.

Extraction of ferredoxin

All extraction and purification procedures were carried out at 4°C. Leaves were ground in a pestle and mortar with a suitable volume of 20mM-Tris–HCl buffer (pH7.4) containing 0.035M-NaCl. Sand was added to facilitate grinding, and polyvinylpyrrolidine was added to adsorb polyphenol oxidase.

After squeezing through two layers of 40-mesh nylon, the homogenate was centrifuged at 40000*g* for 30min. Solid NaCl was added to the clear supernatant to give 0.2M final concentration, and the solution was added to a column (4cm×1cm) of DEAE-cellulose (Whatman DE-23; Whatman Biochemicals Ltd., Maidstone, Kent, U.K.). After washing with 0.2M-NaCl in 20mM-sodium phosphate buffer (pH8.5) the ferredoxin fraction was eluted with 0.8M-NaCl in the same buffer.

The efficiency of the extraction was investigated. Pellets obtained from the initial grinding of greenhouse-grown leaves were re-extracted, and the results indicate that 85–90% of the soluble ferredoxin was removed in the first extraction. For convenience, the tissues were normally extracted once only. A similar 10% loss of ferredoxin occurred on extraction of etiolated bean leaves. It is reasonable to suppose that the same degree of loss occurs upon extraction of leaves at all stages of greening. Ferredoxin values were corrected for the 10% loss during extraction.

In the experiment on the distribution of ferredoxin in etiolated bean plants, homogenates were made from roots, shoots, cotyledons and leaves of 14-day-old dark-grown beans. Pestle-and-mortar grinding could only be used successfully with leaf tissue, since only this had a suitable texture for such an extraction procedure. Cotyledons were homogenized in a Sorvall Omnimixer (Sorvall Inc., Newtown, Conn., U.S.A.) for 10s, speed 6, and roots and shoots were homogenized in an MSE 'Atomix' for 2×15s at full speed.

Bioassay of ferredoxin

O_2 release was measured with a Rank-type O_2 electrode (Rank Bros., Bottisham, Cambs., U.K.), by using P_1S_1 chloroplasts (Whatley & Arnon, 1963) in the presence of limiting amounts of ferredoxin. The reaction mixture (total volume 3ml) consisted of: Tris–HCl buffer (pH7.8), 20μmol; $NADP^+$, 2μmol; P_1S_1 spinach chloroplast suspension, 0.25mg of chlorophyll; and 1560 units of catalase [Boehringer Corp. (London) Ltd., London W.5, U.K.] (1 unit = amount of enzyme catalysing the conversion of 1μmol of substrate/min at 25°C at optimal conditions) to prevent any O_2 uptake caused by Mehler-type reaction (Telfer *et al.*, 1970).

Ferredoxin from spinach, French bean or the blue–green alga *Spirulina* gave identical rates of O_2 release/mol of ferredoxin in the assay system used (cf. Matsubara, 1968). The lower limit of sensitivity of the assay system was 0.1–0.2nmol, and the pH optimum was 8.0, with a sharp decrease in rate of electron transport at higher pH values. The rate of O_2 evolution was proportional to the amount of ferredoxin in the reaction mixture up to 1.5nmol. A standard curve was made, by using purified spinach ferredoxin, for each set of samples assayed.

Assay of ferredoxin by e.p.r. spectroscopy

Because of the lower sensitivity of this method of ferredoxin measurement, it was necessary first to

concentrate semi-purified extracts 5–20-fold on a Sartorius membrane filter (Sartorius Membranfilter G.m.b.H., Göttingen, Germany).

Samples (100μl) were reduced with sodium dithionite solution (final concn. 2mM) for 2min under argon, in tubes matched for internal diameter. The samples were then frozen in liquid N_2. Measurements were made in a Varian E4 e.p.r. spectrometer at 25°K (by using a flow of He gas), or at 77°K (sample immersed in liquid N_2), and the peak-to-peak height of the signal at $g = 1.96$ was taken as a measure of the ferredoxin concentration (see Fig. 1). The lower temperature gave greater signal amplitude and thus greater sensitivity, although similar values for ferredoxin concentration were obtained at both temperatures. Calibration curves were drawn from the signal sizes given by samples of purified spinach ferredoxin. In view of the obvious difficulties in obtaining reproducible signal sizes from dilute samples, precautions were taken to ensure the accuracy of the measurements. Duplicates of each sample were prepared and the signal of each was measured twice. The sensitivity of this assay method was lower than that of the chloroplast bioassay, and only amounts of ferredoxin in excess of 2μmol could reasonably be measured.

Chlorophyll measurement

Chlorophyll was measured in acetone extracts by the method of Arnon (1949).

Results and Discussion

Distribution of ferredoxin in etiolated bean plants

Homogenates were prepared from roots, shoots, leaves and cotyledons of 14-day-old etiolated bean plants as described in the Experimental section. Ferredoxin in these extracts was assayed both by the chloroplast bioassay and by an e.p.r. method, the results being shown in Table 1. Within the sensitivity limits of the assay methods, no ferredoxin was detected in roots or shoots. A small but consistent trace was found in the cotyledons, but the amount appeared to vary in different experiments. The only

Table 1. *Distribution of ferredoxin in etiolated bean*

The values represent the means of at least two determinations from each extract. For e.p.r. spectrometer settings see legend to Fig. 1.

	Ferredoxin content (nmol/g fresh wt.)		
Tissue	Bioassay	E.p.r. at 77°K	E.p.r. at 25°K
Leaf	1.76	1.65	1.42
Cotyledon	0.029	0.097	0.097
Root	<0.01	<0.01	<0.01
Shoot	<0.01	<0.01	<0.01

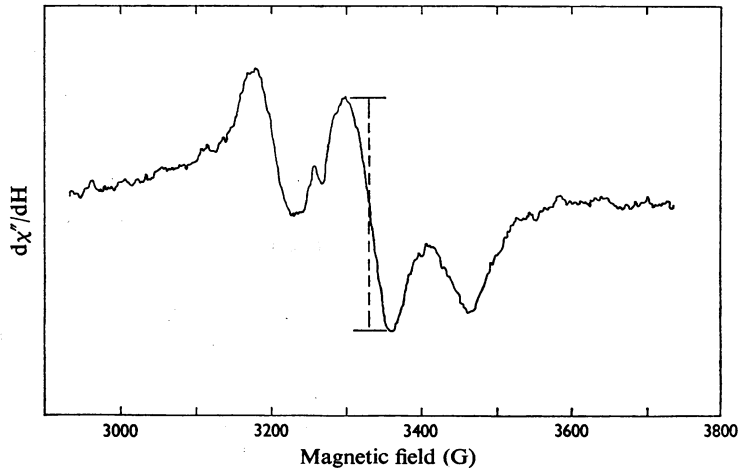

Fig. 1. *E.p.r. spectrum of 20-fold concentrated extract of etiolated bean leaves, prepared and reduced with sodium dithionite as described in the text*

The quantity of ferredoxin was determined by measuring the peak-to-peak distance of the principal signal (g_y = 1.96), as indicated, and comparing it with a standard sample. The instrument settings were: temperature, 26°K; microwave frequency, 9.17GHz; modulation amplitude, 10G. The sample was cooled by means of a stream of cold He gas, and the temperature was measured by a thermocouple placed immediately below the sample tube. The ordinate is the first derivative of the microwave absorption in arbitrary units.

tissue found to contain appreciable quantities of ferredoxin was the leaf. From five determinations by the bioassay, etiolated bean leaves were found to contain 0.104±0.031 (S.D.) nmol per leaf.

Measurements on a single batch of plants indicate that this variation is principally due to physiological differences in growth of the plants in different experiments.

Table 1 shows that good agreement occurs between this method of determination and the e.p.r. method. It is concluded that the semi-purified bean ferredoxin extract does not contain substances interfering with the rate of electron transport in the chloroplast bioassay. To check that the lack of ferredoxin in roots and shoots was not the result of breakdown during extraction, pure spinach ferredoxin was added to one-half of the initial homogenate of each tissue extracted. Recovery of the added ferredoxin was more than 90% from all tissues. In all further experiments reported here, the bioassay only was used.

The fact that ferredoxin is not found in major amounts outside the leaves suggests that it only functions as a photosynthetic reductant and is not involved in other electron-transfer processes, such as nitrite reduction, outside the leaf (Losada & Paneque, 1966). However, this does not exclude the possibility that a ferredoxin may function in the cytoplasm of the leaf cell. The fact that ferredoxin occurs in cotyledons in small amounts is understandable, since rudimentary plastids occur in them, and chlorophyll forms in this tissue on illumination of etiolated plants.

Development of ferredoxin during greening

Fig. 2 shows the development of chlorophyll and ferredoxin during illumination for 50h at high and low light intensities. Chlorophyll development shows a lag of approx. 5h from the onset of illumination, after which the rate of synthesis increases, reaching a maximum at 40h. Ferredoxin development shows no such lag, and the rate of synthesis remains constant throughout the period of illumination. This pattern is reflected in the changing chlorophyll/ferredoxin ratio.

At low light intensity the general pattern is repeated, but the development of chlorophyll and ferredoxin proceeds more slowly. The rate of chlorophyll synthesis is much slower in such low-light conditions, causing the chlorophyll/ferredoxin ratio to increase at a lower rate. Experiments performed at light intensities above 2500lx gave results that were not significantly different from those at 2500lx.

If the period of greening was extended, the net synthesis of both chlorophyll and ferredoxin levelled off after 50–70h. At this time, the chlorophyll/ferredoxin ratio reached a constant value, which was maintained until 160h. This value was measured by the bioassay to be 356±40. This value compares well with the estimate by Tagawa & Arnon (1962) of 400 for both leaves and chloroplasts. If the experiment was performed at a low intensity (60lx) net synthesis of chlorophyll and ferredoxin continued for a longer period and the final quantities obtained were lower. However, the chlorophyll/ferredoxin ratio again reached 358±40.

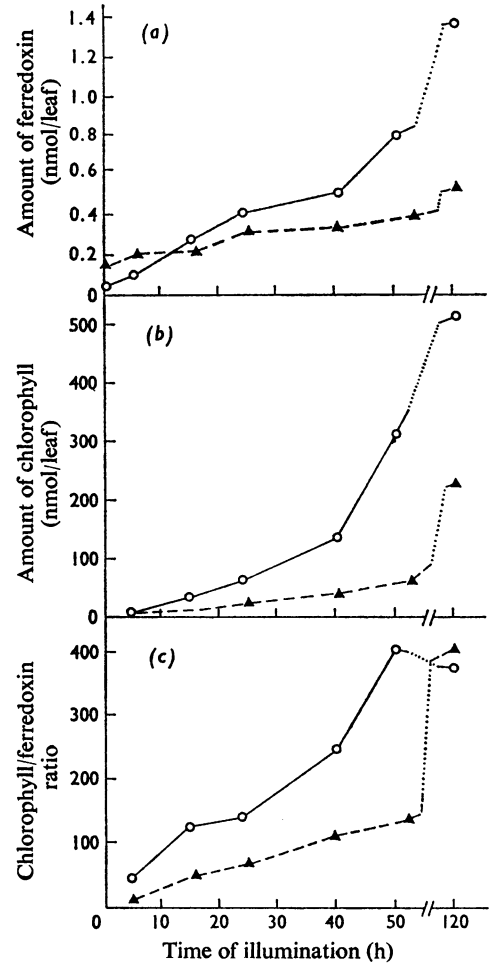

Fig. 2. *Development of (a) ferredoxin, (b) chlorophyll and (c) chlorophyll/ferredoxin ratio during continuous illumination at* 2500*lx* (○) *and* 60*lx* (▲)

For details see the text. The temperature was 25°C in both cases.

The independence of chlorophyll synthesis and ferredoxin synthesis during the early stages of greening is in general agreement with the work of others (Melandri *et al.*, 1967; Phung Nhu Hung *et al.*, 1970; Hudock & Levine, 1964). Hudock & Levine (1964) noted a lag in ferredoxin development

during illumination of synchronously grown *C. reinhardii* (strain Y-2), but it is possible that the changes that occur during darkening and re-greening, such as those that they studied, may not be the same as the changes that occur during the greening of etiolated leaves.

The observation that the final chlorophyll/ferredoxin ratio attains a constant value irrespective of the light intensity suggests that the mature chloroplast contains a fixed number of components per electron-transport chain. The way in which this final stoicheiometry is reached is unknown.

If the quantity of ferredoxin is expressed relative to the area of chloroplast membrane during greening (Fig. 3), the ferredoxin synthesis appears first to exceed, and later to become slower than, the rate of lamellar synthesis.

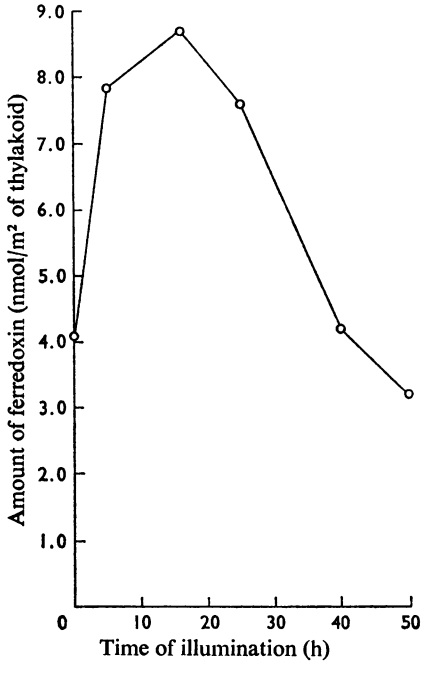

Fig. 3. *Changes in the ferredoxins per unit area of thylakoid membrane during continuous illumination of* 14*-day-old etiolated bean leaves*

For details see the text. Light intensity, 2500lx; temperature, 25°C.

At the same time, Gregory & Bradbeer (1973) have noted an inhibition of thylakoid formation during greening in the presence of D-*threo*-chloramphenicol, whereas in the present work we note a stimulation of ferredoxin synthesis by the same antibiotic (see Table 4).

These observations on the relationship between ferredoxin and thylakoid membrane show that there is no fixed relationship between these components during greening. This implies that the thylakoid membrane is not instrumental in determining the stoicheiometry between chlorophyll and ferredoxin seen during later stages of greening.

Light appeared to be essential for the maintenance of a high rate of ferredoxin synthesis, as shown in Table 2, where etiolated plants were illuminated for 5h and then either replaced in darkness or illuminated for a further 20h. Although no net synthesis in the dark is shown in Table 2, a low rate of ferredoxin synthesis has been noted in other experiments, but it was always less than 5% of the light-dependent rate.

Wavelengths of light inducing ferredoxin synthesis

The 14-day-old etiolated plants were exposed to a short period of illumination at different wavelengths at 24h intervals on each of 3 successive days, the ferredoxin being extracted on the fourth day, 24h after the last illumination. The illumination system was that used by Bradbeer (1971).

The results shown in Table 3 indicate that red light is the most effective in promoting the synthesis of ferredoxin. Blue and far-red light are relatively ineffective, whereas red illumination followed immediately by far-red light gives essentially the same result as red light alone.

Bradbeer (1971) has discussed the role of different light wavelengths in promoting the synthesis of some of the photosynthetic carbon-cycle enzymes and recognizes four distinct responses: (1) a phytochrome response; (2) a non-photoreversible red-light response; (3) a loss of activity owing to blue light; (4) a positive response to red and blue light.

Table 2. *Effect of return to darkness after initial illumination on development of ferredoxin and chlorophyll*

Illumination was at 2500lx and 25°C. The values represent the means of two experiments.

Treatment	Ferredoxin content per leaf (nmol)	Chlorophyll content per leaf (nmol)	Chlorophyll/ferredoxin ratio
Before illumination	0.089	0	—
5h illumination	0.30	9.33	31.1
25h illumination	0.812	147.6	181.0
5h illumination, then 20h darkness	0.189	6.17	32.6

Ferredoxin thus falls into the second category. The nature of the red-light photoreceptor is unknown, but could be protochlorophyllide, which is known to absorb light for its own photoreduction. Eytan & Ohad (1972) have suggested that chlorophyll synthesis may influence the synthesis of chloroplast proteins.

Site of ferredoxin synthesis

Experiments were carried out with the protein-synthesis inhibitors lincomycin (Ellis & Hartley, 1971) and D-*threo*-chloramphenicol, which interact specifically with the 70S ribosomes of chloroplasts. Control experiments were also carried out in which the stereoisomer L-*threo*-chloramphenicol was used (Ireland & Bradbeer, 1971) to compensate for the non-specific effects on growth shown by the D-*threo* isomer. This L-*threo* isomer has no specific effects on protein synthesis, but shows the same non-specific effects as the D-*threo* form.

The 14-day-old etiolated plants were cut at the hypocotyl and the cut ends placed in the inhibitor solution at the beginning of the period of illumination. After 6h, the amount of inhibitor taken up by the plants was calculated from the volume of solution absorbed, and the plants were transferred to water. After a total period of 50h illumination, the ferredoxin was extracted from the leaves as described in the Experimental section. None of the inhibitors used was found to affect the rate of electron transport in the bioassay system.

The results are given in Table 4, which shows that there was a significant stimulation of ferredoxin development by both lincomycin and D-*threo*-chloramphenicol (in comparison with the L-*threo* isomer). This suggests that ferredoxin is synthesized on cytoplasmic ribosomes rather than chloroplast ribosomes. The observed stimulation of ferredoxin synthesis may be due to a greater amount of protein precursors being made available as a result of the inhibition of protein synthesis in the chloroplasts.

Armstrong *et al.* (1971) found that specific inhibitors of chloroplast translation (spectinomycin and D-*threo*-chloramphenicol) did not affect the development of ferredoxin during greening of *C. reinhardii*. However, such development was inhibited by cycloheximide, which specifically inhibits translation on cytoplasmic ribosomes. They concluded that the site of translation of ferredoxin was the 70S ribosomes of chloroplasts. The present paper agrees with these conclusions.

Armstrong *et al.* (1971) also studied the effect of rifampicin (Hartmann *et al.*, 1967) on the develop-

Table 3. *Effect of short light treatments on development of ferredoxin and chlorophyll*

The effects of red, far-red and white light were investigated in one experiment, and the effects of blue and blue+red light were examined in a separate experiment. The results represent the means of two experiments in each case. Light intensities used are those described by Bradbeer (1971).

Treatment	Ferredoxin content per leaf (nmol)	Chlorophyll content per leaf (nmol)
(1) Before illumination	0.0678	0
Dark control	0.090	0
Red	0.201	2.18
Far red	0.120	1.44
Red/far red	0.189	2.13
White	0.272	2.42
(2) Before illumination	0.206	0
Dark control	0.266	0
Blue	0.294	1.89
Red/blue	0.317	2.51

Table 4. *Effect of inhibitors of protein synthesis on development of ferredoxin and chlorophyll*

D- and L-*threo*-Chloramphenicol were used at a concentration of 1 mg/ml. After the period of inhibitor uptake, the concentration per leaf was approx. 200 μg. Lincomycin was used at a concentration of 50 μg/ml. After the period of inhibitor uptake, the concentration per leaf reached approx. 9 μg. Plants were illuminated for a total of 50h in each case (2500lx, 25°C).

Treatment	Ferredoxin content per leaf (nmol)	Chlorophyll content per leaf (nmol)	Chlorophyll/ferredoxin ratio
Before illumination	0.104	0	—
Water	0.90	202.0	224.0
L-*threo*-Chloramphenicol	0.76	99.0	130.0
D-*threo*-Chloramphenicol	1.12*	40.6†	40.7
Lincomycin	2.16	148.6	68.5

* Significantly different from L-*threo*-chloramphenicol at $P = 0.05$.
† Significantly different from L-*threo*-chloramphenicol at $P = 0.01$ as determined by Student's t test.

ment of ferredoxin in *C. reinhardii*. This is a specific inhibitor of chloroplast DNA-dependent RNA polymerase in this alga, and can be used to determine the site of transcription of proteins. They noted no effect of this inhibitor on the development of ferredoxin, and concluded that the nucleus contains the genetic information for ferredoxin synthesis. We have examined the effects of rifampicin in greening bean leaves, and find only a general inhibition of photosynthetic CO_2 fixation and electron transport. We conclude that rifampicin has no specificity for the chloroplast RNA polymerase in this plant. Bottomley *et al.* (1971) have also shown this lack of specificity in higher plants. Bradbeer (1970) suggests a correlation between the site of synthesis and the presence of a lag phase during development, noting that chloroplasts require a period of activation before being able to perform any protein synthesis, and the present work agrees with this proposal in that ferredoxin development has no lag period, while it is synthesized on cytoplasmic ribosomes.

We cannot discount the possibility that ferredoxin development is to some degree due to the addition of iron–sulphur groups to existing apoprotein. However, although the inhibitor of cytoplasmic protein synthesis, cycloheximide, was not used in the present paper, Armstrong *et al.* (1971) have obtained inhibition of ferredoxin synthesis in *C. reinhardii* with this antibiotic.

In addition, etiolated leaves are known to synthesize protein when exposed to continuous illumination and there are a number of reports in which increases in enzyme activity during greening have been correlated with actual synthesis of the enzymes (e.g. Lyttleton, 1956).

Many problems remain to be solved in relation to the development of ferredoxin and other chloroplast electron-transport proteins. Among these is the question of transport of such relatively large molecules across the fairly impermeable chloroplast membrane. There is also the question of how the synthesis of these molecules is correlated since, as shown in the present paper, there must be an underlying control mechanism.

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