Light Modulation of the Activity of Protochlorophyllide Reductase

Richard E. MAPLESTON and W. Trevor GRIFFITHS
Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

(Received 3 December 1979)

Illumination of etiolated plants effects the activity of protochlorophyllide reductase (NADPH–protochlorophyllide oxidoreductase) in the plastids. Constant illumination or a 2-min light-triggering of etiolated plants leads to an approx. 80% decrease in activity of the enzyme, a change that can be reversed by returning the plants to darkness. The change in activity results from an alteration of the $V_{\max}$, rather than K_m . Despite the fact that exogenous pigments effect the activity of the enzyme *in vitro*, no correlation could be drawn between the concentrations of pigments *in vivo* and activity of the enzyme.

It is becoming increasingly apparent that light plays an important role in the regulation of certain biochemical processes. Not surprisingly, the majority of these events are found in the Plant Kingdom both in higher plants (see, e.g., Bradbeer, 1976; Lendzian & Bassham, 1975; Wolosiuk & Buchanan, 1977; Anderson & Avron, 1976; Champigny & Bismuth, 1976; Bennett, 1977) and lower plants (O'Brien & Powls, 1974). Such processes are, however, by no means confined to plants, since in fact one of the best-characterized light-regulated processes is that of the cyclic GMP phosphodiesterase of vertebrate photoreceptors (Wheeler & Bittensky, 1977).

In plants, the majority of light-modulated reactions are only indirectly influenced by light, as the primary event is to excite photosynthetic electron transfer, which results in the generation of active effector molecules. By influencing the activity of certain enzymes, these substances afford mechanisms for regulating photosynthetic CO_2 fixation. Another important role of light in the regulation of plant metabolism concerns etioplast development. Here light appears to act through two distinct mechanisms involving different photoreceptors, namely phytochrome, which may control a range of developmental processes (Smith, 1977), and protochlorophyllide, the light-dependent reduction of which produces chlorophyll, an obligate prerequisite for chloroplast development.

Protochlorophyllide photoreduction is made possible as a consequence of the activity of the enzyme protochlorophyllide reductase (NADPH–protochlorophyllide oxidoreductase) (Griffiths, 1978), which forms a photoactive complex with protochlorophyllide and NADPH. The enzyme thus

performs a key role during chloroplast development and its activity may well be subject to light regulation. In the present paper our unexpected findings on the effect of light on the activity of protochlorophyllide reductase are described. It is shown that illumination of dark-grown plants leads to a dramatic decrease in the activity of the enzyme in the plastids, a process that can be reversed by returning the plants to darkness. Changes in pigment concentrations induced by illumination of etiolated plants have been studied and also the effects of addition of various pigments on the enzymes' activity *in vitro* have been investigated in attempts to explain the changes in the activity of the enzyme. Although the real significance of the illumination-induced changes in reductase activity are not completely understood, it is possible that they are involved in some subtle way in regulating chlorophyll formation. A preliminary report of some of this work has already appeared (Mapleston & Griffiths, 1977).

Materials and Methods

Biological material

Etiolated 7-day-old barley (*Hordeum vulgare* L., cultivar Proctor) and wheat (*Triticum sativum* L., cultivar Capella) seedlings were cultivated as previously described (Griffiths, 1975). Illumination of the seedlings was achieved by placing the growth trays under a bank of white fluorescent tubes supplemented with 40W tungsten lamps giving an intensity at the plant surface of $0.87 \text{ mW} \cdot \text{cm}^{-2}$.

Etioplasts were isolated from the excised plant tissue by homogenization in isolation buffer (Griffiths, 1975) (approx. 1 g of tissue/2 ml of buffer) for 10 s with a Polytron homogenizer set at maximum speed. The resulting brei was squeezed

Abbreviation used: Mes, 4-morpholine-ethanesulphonic acid.

through two layers of muslin and four layers of Miracloth and the etioplast fraction recovered by differential centrifugation as previously described (Griffiths, 1975). The etioplast pellets were resuspended and stored in darkness on ice before use. Supplementation of the resuspending medium with 5 mM-cysteine had been found to stabilize the enzyme in isolated preparations and was therefore routinely added during the present work.

Assay of protochlorophyllide reductase activity

The enzyme was assayed in the plastid preparations by continuously monitoring chlorophyllide synthesis with the flash-illumination assay described previously (Griffiths, 1978). An optimum concentration of Triton X-100 was included in all the assays to facilitate the entry of substrates to the enzyme with the exact amount of Triton to be added being predetermined for each preparation. Chlorophyllide formation under continuous illumination was achieved under identical conditions, except that illumination was now provided by a 55 W quartz/halogen lamp illuminating the sample from one side and through an interference filter (Balzers B40) transmitting maximally at 440 nm. Light intensity was varied in these experiments by means of neutral-density filters of known transmission inserted into the light beam. To ensure that the enzyme was being assayed under optimum conditions, linearity between the measured rate and amount of protein added was always ascertained by varying the latter. In the present work a unit of reductase activity is defined as a rate of chlorophyllide synthesis of 1 nmol/min measured under standard assay conditions.

Isolation and purification of pigments

Protochlorophyllide was recovered from the total lipid fraction of etiolated barley plants by the phase-separation technique described previously (Griffiths, 1978).

Chlorophyll *a* and chlorophyll *b* were purified by preparative t.l.c. from the total lipid fraction prepared from spinach (*Spinacia oleracea*) leaves. The technique was adapted from that used previously for isolating protochlorophyll from etiolated barley and involved the initial separation of the total lipid on plates coated with a 0.5 mm layer of adsorbent prepared by mixing 24 g of Kieselguhr, 6 g of Kieselgur G, 6 g of powdered Analytical-grade CaCO_3 and 36 mg of $\text{Ca}(\text{OH})_2$ in 84.5 ml of 5 mM-potassium ascorbate, pH 7.2. The developing solvent was light petroleum (b.p. 60–80°C)/propan-2-ol/water (400:20:1, v/v). In this system, chlorophylls *a* and *b* were well separated and migrated with R_F values of 0.75 and 0.60 respectively. Both were eluted separately into ether and rechromatographed on further 0.5 mm thin layers of cellulose prepared

from 45 g of cellulose (Whatman) suspended in 100 ml of water. The developing solvent in this case was light petroleum/acetone/propan-1-ol (1800:200:9, v/v). The R_F of chlorophyll *b* in this system was 0.55, whereas chlorophyll *a* migrated with an R_F of approx. 0.40. The two chlorophylls were eluted from the adsorbent into ether, concentrated and stored at -20°C under N_2 .

Chlorophyllide *a*, i.e. the free acid corresponding to esterified chlorophyll *a*, was prepared from the latter by enzymic hydrolysis with the enzyme chlorophyllase. A crude preparation of chlorophyllase was prepared from young fresh spinach leaves as described by McFeeters (1975). Enzymic hydrolysis of the chlorophyll *a* was carried out by a method adapted from the chlorophyllase assay procedure described by McFeeters *et al.* (1971). In a typical preparation, 1.5 ml of purified 2.5 mM chlorophyll *a* in ether was incubated with 5 ml of chlorophyllase and 23.25 ml of buffer comprising 80 mM-potassium phosphate 12 mM-sodium acetate, 12 mM-sodium borate, pH 7.6, and adjusted to an ionic strength of 0.3 with KCl. Triton X-100 at a final concentration of 0.24% (w/v) was included in the incubation mixture. After incubation in darkness for 16 h at 30°C in a shaking water bath, the mixture was quenched with 1 vol. of aqueous KOH, 6 vol. of hexane and 4 vol. of acetone. The concentration of KOH was that which raised the pH of an equal volume of reaction mixture to 8.5. This treatment resulted in the formation of two phases, the uppermost hexane layer containing non-hydrolysed chlorophyll *a*, which was discarded, and a lower aqueous acetone phase containing the chlorophyllide *a*. The chlorophyllide *a* was extracted from the aqueous phase into diethyl ether, washed several times with water, dried over anhydrous Na_2SO_4 and concentrated under N_2 . Final purification of the chlorophyllide *a* was carried out by preparative (0.5 mm thick layer) t.l.c. using plates coated with an adsorbent of 30 g of cellulose (MN 300, Macherey Nagel and Co., Düren, Germany), suspended in 175 ml of water and developed with methanol/methylene chloride/water (40:14:5, by vol.). In this system, chlorophyllide *a* migrated with an R_F of approx. 0.9 and was recovered by extraction into diethyl ether and stored, concentrated under N_2 at -20°C . A final yield of chlorophyllide *a* produced by this procedure was 425 nmol, representing 11.3% of the starting chlorophyll *a*. Despite this low yield, this enzyme procedure was infinitely better than alkali hydrolysis, owing to the large number of chemically altered chlorophyll products resulting from the latter, even under the mildest conditions.

Results

The enzyme protochlorophyllide reductase is

readily assayed in isolated etioplasts of dark-grown barley plants (Griffiths, 1978). The reliability of the assay was ascertained by isolating four preparations of plastids from samples of the same etiolated-barley starting material. On assay, protochlorophyllide reductase specific-activity values of 1.48, 1.40, 1.39 and 1.52 units/mg of protein were obtained giving a mean value of 1.46 and an S.D. of 0.05. These values indicate the high degree of reproducibility of the assay. However, it has to be stated that such reliable values can only be guaranteed for plants originating from a single planting and all grown under identical conditions. Plants grown under apparently similar conditions but on different occasions produce slightly different values for protochlorophyllide reductase activities, since factors that are difficult to control precisely, such as age, relative humidity, temperature etc., do influence the activity of the enzyme. It is important therefore that, in experiments designed to study the effects on the reductase of varying experimental parameters such as illumination, only plants taken from the same harvest should be used for the different treatments to ensure strict uniformity of experimental material. This ruling was adhered to in all the work discussed here.

To study the effects of illumination on the activity of the enzyme, plastids were isolated from plants pre-illuminated for various time periods before harvest. The results obtained are plotted in Fig. 1, and indicate that, contrary to what might have been expected, a rapid decrease in the specific activity of the enzyme occurs on illumination of the plants. The enzyme in plastids from dark-grown plants had an activity of 1.1 units/mg of protein: after 30 min illumination the activity decreased to 0.2 unit/mg of protein, and remained at this value during a further 30 min in the light. Increasing the period of illumination (results not shown) up to 6 h resulted in only a very slight further decrease in activity. The activity of the reductase in plastids from illuminated plants, despite being low, was quite stable for up to 6 h in plastids kept on ice in a buffer supplemented with cysteine. Beyond this time, however, the activity declined quite rapidly.

The effect of light-triggering (2 min illumination) of dark-grown plants on the activity of the reductase in subsequently isolated plastids is shown in Fig. 2. In this experiment, plastids were isolated from dark-grown plants before any illumination and the reductase was assayed. After 2 min illumination of the remaining plants, plastids were again isolated and assayed for reductase activity. Finally the plants were returned to darkness and the plastid isolation and assay procedures repeated on samples withdrawn after various periods of redarkening. In this experiment (Fig. 2), plastids from non-illuminated plants had a protochlorophyllide reductase activity

Fig. 1. *Effect of continuous pre-harvest illumination on protochlorophyllide reductase activity in the subsequently isolated plastids*

Etioplasts were isolated from dark-grown plants before and after illumination for different lengths of time. Protochlorophyllide reductase activity was assayed in the plastids immediately after isolation by using the standard assay procedure.

of 3.35 units/mg of protein. Plants illuminated for 2 min yielded plastids with an activity of 2.03 units/mg. This decrease continued in darkness to give a minimum value of 0.78 unit/mg after 60 min in darkness, indicating that continuous light is not required for a decrease in enzymic activity. Fig. 2 also shows that, after a long period in darkness after illumination, the initial decrease in activity of the enzyme is followed by a rise in enzyme activity (see also below).

A preliminary experiment had indicated that, if briefly illuminated plants are returned to darkness, then recovery of the protochlorophyllide reductase activity occurs. Fig. 3 shows the time course of this recovery as it occurs after transferring 30 min-illuminated plants back into darkness. Immediately after the illumination the plants produced etioplasts with the characteristically low protochlorophyllide reductase activity of 0.2 unit/mg of protein. On returning the plants to darkness this value gradually increased, reaching an activity of 1.1 units/mg after 3 h in darkness, this final value being typical of the activity normally associated with preparations from

Fig. 2. Effect of a pre-harvest brief illumination period followed by darkness on protochlorophyllide reductase activity in subsequently isolated plastids

Dark-grown barley plants were briefly illuminated for 2 min (■) and returned to darkness for a further 4 h, during which time etioplasts were isolated and assayed for protochlorophyllide reductase activity.

dark-grown tissues. It appears, therefore, that protochlorophyllide reductase in etioplasts undergoes a decrease in activity on continuous or brief illumination of the plants and that, furthermore, this decrease is reversed by returning the plants to darkness.

The data in Fig. 4 show the effect of varying the period of illumination on the extent of re-activation of the reductase during a subsequent 3 h period of darkness. A 30 and a 60 min period of illumination followed by 3 h in darkness gave roughly similar activities for the enzyme of approx. 1.2 units/mg of protein. However, increasing the time of illumination to 3 and 6 h respectively gave progressively lowered re-activation of the enzyme, resulting in activities of 0.55 and 0.35 unit/mg of protein respectively.

To check whether these phenomena are peculiar to etiolated barley or whether they are of a more widespread occurrence, some investigations were carried out on etiolated wheat plants. These were

Fig. 3. Effect of re-darkening of illuminated plants on plastid protochlorophyllide reductase activity

Dark-grown plants were illuminated for 30 min and subsequently returned to darkness for various time intervals, after which plastids were isolated and assayed for activity of the enzyme.

Fig. 4. Effect of length of illumination period on the subsequent extent of recovery of protochlorophyllide reductase activity in darkness

Dark-grown barley plants were illuminated for different lengths of time and returned to darkness for 3 h, after which plastids were isolated and assayed for the activity of protochlorophyllide reductase.

Table 1. *Effect of pre-harvest illumination and return to darkness on protochlorophyllide reductase in dark-grown wheat plastids*

Enzymic activity was estimated by the normal assay procedure in plastids isolated from 7-day-old wheat plants which were subjected to treatments with light and darkness.

Sample	Protochlorophyllide reductase activity (units/mg of protein)
Etioplasts	1.14
Plastids from plants illuminated for 40 min	0.42
Plastids from plants illuminated for 40 min and returned to darkness for 3 h	1.17

subjected to the same treatment already given to barley with etioplasts isolated from dark-grown plants before and after a 40 min illumination period and from some returned to darkness for 3 h after illumination. The activities of protochlorophyllide reductase in the resulting plastids (Table 1) show that 40 min illumination of wheat plants, as in barley, leads to a decrease in reductase activity from 1.14 to 0.42 unit(s)/mg of protein. Returning the illuminated plants to darkness for 3 h gave an increase in the activity of the enzyme to 1.17 units/mg of protein, thereby mimicking exactly the behaviour of the barley enzyme.

Some properties of the enzyme in illuminated plants were next studied in order to gain some insight into the mechanism of de-activation. The $V_{\max}$ and K_m (protochlorophyllide) values were estimated for the enzyme in plastids from dark and 30 min-illuminated plants. The resulting Lineweaver-Burk plots are shown in Fig. 5, which indicate $V_{\max}$ values for the dark and illuminated preparations of 0.66 and 0.078 unit/mg of protein respectively, whereas the corresponding extrapolated K_m values were 0.103 and 0.108 μM respectively. The decrease in activity of the enzyme on illumination is thus due to a decrease in $V_{\max}$, rather than an increase in K_m . Though similar data for the second substrate for the enzyme, NADPH, are not available, it is also unlikely that the K_m for this substrate changes during illumination, since increasing the NADPH concentration in assay mixtures failed to increase the reductase activity of the enzyme in plastids from illuminated plants (R. E. Mapleston, unpublished work).

It was considered that the change in reductase activity seen on illumination might have resulted from an illumination-induced change in the concentration of some effector molecule. Protochlorophyllide and chlorophyll(ide) are compounds whose concentration in plants are known to be effected by illumination. The effect of illumination on the amounts of these pigments in barley plants was therefore estimated to try and see if any correlation

Fig. 5. *Double-reciprocal plots of protochlorophyllide reductase activity versus protochlorophyllide concentration in plastids from etiolated and illuminated plants* Plastids were isolated from dark-grown (●) or 35 min-illuminated (○) plants and reductase activity determined at various protochlorophyllide concentrations in an assay mixture of 2.5 ml of 'isolation buffer', 0.12 mM-NADPH (as a regenerating system) and 200 μM -Triton X-100.

could be drawn between these and the activity of protochlorophyllide reductase.

Fig. 6 records the changes in amounts of chlorophyll and chlorophyllide *a* in 7-day-old etiolated barley shoots exposed to constant illumination for up to 3 h. There is an initial rapid increase in chlorophyllide *a* from 0 to 4 nmol/ten shoots on

illumination, followed by a much slower decrease to a low value of about 0.1 nmol/ten shoots over the next 20 min, at which value it remains for the next 3 h or so of illumination. The decrease in chlorophyllide *a* is accompanied by a concomitant increase in the amount of chlorophyll *a* during the initial 20 min of illumination, and this is followed by a lag of about 1 h before the commencement of the rapid phase of chlorophyll synthesis, which continues for the next 3 h. Recalling the data describing the decline in reductase activity during 30 min of illumination (Fig. 1), it is obvious that neither chlorophyll *a* nor chlorophyllide *a* amounts can be equated with activity of the enzyme.

It has already been mentioned (Fig. 4) that the extent of re-activation of the reductase enzyme in darkness after variable periods of illumination decreases as the period of illumination is increased.

Fig. 6. Changes in pigment concentrations during continuous illumination of dark-grown plants

Dark-grown plants were illuminated for various time intervals, after which samples were taken and chlorophyll *a* (O) and chlorophyllide *a* (●) were extracted and assayed as described by Treffry (1970).

Fig. 7 indicates that similar behaviour is displayed by the pigment protochlorophyllide. Thus, after an illumination period of 1 min, which has decreased the amount of protochlorophyllide to zero, the pigment is resynthesized in darkness until after 3 h it has reached the extent of 7.8 nmol/ten shoots. As the

Fig. 7. Effect of length of illumination of dark-grown plants on the extent of subsequent accumulation of protochlorophyllide in darkness

Dark-grown barley plants were illuminated for 1 (Δ), 60 (●) or 210 (○) min and returned to darkness. At various intervals during the re-darkening, protochlorophyllide was extracted and assayed as described by Treffry (1970).

Table 2. Effect of aminolaevulinate feeding on the re-activation of protochlorophyllide reductase in illuminated plants returned to darkness

Dark-grown plants were illuminated for 5 h and a sample used for plastid isolation. Of the remainder some were placed in water and the rest in a solution containing 10 mM-aminolaevulinate/1 mM-Mes, pH 6.8, and both incubated in darkness in a current of air. After 3 h in darkness plastids were isolated from both samples in the usual manner. Plastid preparations were assayed for protochlorophyllide reductase activities. Ten plants were also taken from each sample and the protochlorophyllide content determined by pigment extraction.

Treatment	Protochlorophyllide reductase activity (units/mg of protein)	Protochlorophyllide (nmol/ten shoots)
5 h Illumination	0.156	0
5 h Illumination plus 3 h in darkness in water	0.283	2.18
5 h Illumination plus 3 h in darkness in aminolaevulinate	0.278	33

illumination time is increased so also decreases the amount of protochlorophyllide re-accumulated in 3 h of subsequent darkness; e.g., after 3.5 h of illumination only, 2.1 nmol of protochlorophyllide/ten shoots is re-accumulated; these data are in accord with previous observations by Sundquist (1974).

It is possible to elevate the amount of protochlorophyllide in a plant by feeding with 5-aminolaevulinate, thus providing further means of checking the relationship between protochlorophyllide accumulated and activity of the reductase. Table 2 records the effects on amount of protochlorophyllide and protochlorophyllide reductase activities of feeding 5-aminolaevulinate in darkness for 3 h to plants pre-illuminated for 5 h. Feeding the porphyrin precursor leads to a 15-fold increase in the amount of protochlorophyllide compared with the control unsupplemented plants. However, despite this, the reductase activity in the 5-aminolaevulinate-fed and control plants were very similar at 0.278 and 0.283 unit/mg of protein respectively.

With the isolated samples of purified chlorophyllide *a* and chlorophyll *a* it was possible to study the effects of these pigments on the activity of protochlorophyllide reductase *in vitro*. For these studies the pigments were solubilized in cholate in a manner identical with that used for solubilizing protochlorophyllide (see the Materials and Methods section). The effects of adding increasing concentrations of these two pigments on reductase activities are recorded in Fig. 8. It is apparent that both pigments inhibit protochlorophyllide reductase to various extents, with chlorophyllide *a* being the

most effective. From the plots, the concentration of pigment required to decrease the reductase activity by 50% was respectively 1.0 μM for chlorophyllide *a* and 2.5 μM for chlorophyll *a*. In similar experiments (results not shown) chlorophyll *b* at 3.75 μM also gave a 50% inhibition of the activity of the reductase.

Discussion

The etioplast in dark-grown higher plants represents a state of dark-arrested chloroplast development in as much as illumination brings about rapid formation of mature chloroplasts. Light seems to function by activating various biosynthetic processes by synthesis *de novo* of new enzymes or by activation of pre-existing latent enzymes. The literature contains many reports dealing with activation or synthesis, during greening, of enzymes involved with most major biosynthetic processes such as protein (Ellis, 1977) and nucleic acid synthesis (Bogorad, 1973; Apel & Kloppstech, 1978), carbohydrate synthesis (Bradbeer, 1973), and lipid synthesis (Appelqvist *et al.*, 1968; Tremolieres & Lepage, 1971). The most obvious consequence of greening is the synthesis of chlorophyll, this pathway remaining latent in dark-grown plants through the inactivity or absence of enzymes responsible for 5-aminolaevulinate synthesis, together with the inability to phototransform protochlorophyllide.

Illumination, besides promoting these two reactions, also enhances the activities of other enzymes involved with chlorophyll formation. Among the best documented of these are 5-aminolaevulinate dehydratase (Steer & Gibbs, 1969), porphobilinogen deaminase (Schneider, 1971), and chlorophyllase (Stobart & Thomas, 1968). The decrease in the specific activity of the enzyme protochlorophyllide reductase observed during illumination of etiolated plants in the present work (Fig. 1) was, against this background, rather an unexpected finding.

Either continuous illumination (Fig. 1) or a single brief exposure to light (Fig. 2) followed by a period in darkness consistently resulted in a decrease in the specific activity of the enzyme in barley etioplasts to a value about 10–20% of that found present before illumination. Strictly comparable results were obtained with plastids from similarly treated wheat plants (Table 1), which would suggest that the phenomenon is of general occurrence rather than a peculiarity of etiolated barley. Further testament to the physiological nature of the process is the fact that, in both barley (Fig. 3) and wheat (Table 1), return of the illuminated plants to darkness produces a reactivation of the enzyme to the pre-illuminated value within approx. 3 h.

The activity of protochlorophyllide reductase, therefore, would appear to be inversely correlated

Fig. 8. Effect of exogenous chlorophyll and chlorophyllide *a* on protochlorophyllide reductase activity in isolated etioplasts

Isolated etioplasts were assayed for protochlorophyllide reductase activity after preincubation for 3 min in darkness with various concentrations of cholate-solubilized chlorophyll (O) and chlorophyllide *a* (●).

with the rate of tissue chlorophyll formation, i.e., in darkness, when no chlorophyll formation is possible, the activity of the enzyme is at a maximum, whereas in plants illuminated for, say, 3 h, when the rate of chlorophyll synthesis is at its greatest, then protochlorophyllide reductase activity is very low. However, in considering this apparent paradox it must be recalled that the activity of the enzyme in etioplasts from dark-grown barley plants (>1 nmol/min per mg of protein) is an order of magnitude greater than the mean rate of chlorophyll synthesis achieved by 7-day-old etiolated barley during the first 12 h of illumination, i.e. 0.023 nmol/min per mg of protein (Griffiths, 1978). Therefore one is forced to conclude that even the decreased activity measured in plastids from illuminated plants must still be sufficient for the required biosynthetic flux commensurate with the rate of chlorophyll formation *in vivo*. It must be emphasized also that protochlorophyllide reductase activities *in vitro* are monitored under conditions of saturating substrate (protochlorophyllide and NADPH) concentrations and most probably are not the same as those actually occurring in the intact leaf.

Regarding the factors responsible for the illumination/darkness-induced changes in the activity of protochlorophyllide reductase, several possibilities were considered during the course of the present work. Thus early on it was established that the illumination-induced decrease in activity was not the result of an assay artefact caused by light limitation as a result of shielding by the chlorophyll (Mapleston, 1978). Also, pigment photo-oxidation producing toxic products was eliminated as being responsible for the light-induced inhibition of the enzyme, since the same general behaviour could be observed in red light ($\lambda > 640$ nm), which causes no photo-oxidation of pigments (Castelfranco *et al.*, 1974).

Consideration of the Michaelis parameters of the inhibited enzyme from illuminated plants, i.e., a decreased $V_{\max}$ but an unchanged K_m compared with the active dark enzyme. (Fig. 5), leads to the conclusion that inhibition by light is non-competitive. Isolated chlorophyllide *a* was shown to be an effective inhibitor of the dark enzyme *in vitro* (Fig. 7), and evidence has been presented (Mapleston, 1978) that this inhibition is also non-competitive in nature. The possibility was therefore considered that light-induced chlorophyllide *a* accumulation was responsible for the inhibition *in vivo*. However, it was also found that both chlorophyll *a* (Fig. 7) and chlorophyll *b* (Mapleston, 1978) inhibited protochlorophyllide reductase at very low concentrations, 50% inhibitions being attained with approx. 2.5 and 3.8 μ M respectively, implying, perhaps, non-specific inhibition by these compounds. It is obvious that the inhibition by chlorophylls *a* and *b*

cannot be expressed during plant greening, since plastids from normally grown plants contain concentrations of approx. 5 and 2 mM respectively of these two pigments. In the plastid, however, the pigments are not free, but rather bound as pigment-protein complexes and as such are unavailable, even at low concentration, for inhibition of protochlorophyllide reductase. The fact that all of the chloroplasts' chlorophyll has been shown to be complexed to protein (Markwell *et al.*, 1979) would endorse this proposal.

Regarding chlorophyllide, despite its effectiveness as an inhibitor *in vitro*, the time-course profiles of chlorophyllide concentration *in vivo* (Fig. 6) did not parallel the illumination-induced change in enzyme activity (Fig. 1). During continuous illumination of etiolated plants, the high concentration of chlorophyllide *a* formed immediately by photoconversion of the protochlorophyllide was almost completely depleted as a result of esterification within 20 min and thereafter remained at this low value (Fig. 6). A somewhat similar rate of phytylation has been observed in pea (*Pisum sativum*) seedlings, where 30 min was required for 80% esterification during continuous illumination (Treffry, 1970). In contrast, protochlorophyllide reductase in our experiments was, before illumination, very active, the onset of illumination resulting in a decline in activity (Fig. 4) that continues for 30 min and thereafter remains relatively constant. It is unlikely, therefore, that the changes in chlorophyllide *a* concentration *in vivo* can account for the observed changes in enzyme activity.

Another pigment potentially capable of fulfilling a role as a modulator of protochlorophyllide reductase, in this case an activator, is protochlorophyllide. It is possible that protochlorophyllide might stimulate its own photoconversion, since conditions *in vivo* leading to high reductase activities invariably have associated high protochlorophyllide concentrations. Thus although the disappearance of protochlorophyllide by its own photoconversion is much more rapid than the light-induced de-activation of the enzyme, during continuous illumination, in agreement with the data of Treffry (1970), no protochlorophyllide could be detected (Mapleston, 1978), and here also the enzyme activity is at a minimum. Again, the time course for the re-activation of the reductase in darkness after illumination (Fig. 3) correlates with the rate of protochlorophyllide resynthesis (Mapleston, 1978; Augustinusson & Madsen, 1965). Also, the extent of protochlorophyllide resynthesis after different times of illumination (Mapleston, 1978; Sundqvist, 1974) is strictly paralleled by the differing extents of re-activation of the reductase under similar conditions (Fig. 4). However, on feeding 5-aminolaevulinate, after illumination, the synthesis of protochloro-

phyllide is stimulated 15-fold (Table 3), whereas the enzyme activity remains unaffected by this treatment. Therefore only a casual relationship exists between protochlorophyllide concentration and protochlorophyllide reductase activity. We are therefore forced to conclude that the illumination-induced changes in pigment concentrations cannot account for the corresponding observed changes in protochlorophyllide reductase activity.

A change in protein concentration has been considered in attempting to explain the light-modulated reductase activity. This was prompted by the fact that Sauer and co-workers (Alscher *et al.*, 1976; Canaani & Sauer, 1977) claimed to have identified a cytoplasmically synthesized 15 000-mol.wt. pigment-binding peptide that forms a part of the 'holochrome' unit and that after illumination becomes incorporated along with the associated pigment into the thylakoid membrane. This implied that on formation of the 'holochrome', as the enzyme-substrate complex of protochlorophyllide reductase, the light inhibition of the reductase activity reported here, could, according to Sauer's model, be conceivably due to the depletion of this subunit. Recovery of the enzyme in darkness would then be the result of reaccumulation of the subunit by synthesis *de novo*. However, it has been shown (Maplestone, 1978) that if etiolated barley plants are illuminated for 40 min after treatment with cycloheximide (50 µg/ml) and returned to darkness for 3 h, no effect on the recovery of the reductase activity is observed, implying, contrary to the view of Sauer's group, that stoichiometric provision of a peptide is not required for full activity of the reductase. A similar negative effect was also obtained in chloramphenicol-treated plants.

In conclusion, it appears that the unexpected, but nevertheless noteworthy illumination-induced changes in activity of protochlorophyllide reductase cannot be accounted for by changes in any of the components, pigments or proteins studied here. We have demonstrated (T. Griffiths & R. E. Maplestone, unpublished work) that these changes in the enzyme's activity are a direct consequence of the illumination/darkness-induced alteration of the redox state of the plastid NADP pool.

R. E. M. thanks the Science Research Council for financial support through a Research Studentship. Part of the work was financed by a grant (no. GR/A 72490) from the Science Research Council.

References

- Alscher, R. G., Hawkes, S. P. & Sauer, K. (1976) *Biochem. Biophys. Res. Commun.* **73**, 240–247
- Anderson, L. E. & Avron, M. (1976) *Plant Physiol.* **57**, 209–213
- Apel, K. & Klopstech, K. (1978) *Eur. J. Biochem.* **85**, 581–588
- Appelqvist, L. A., Boynton, J. E., Stumpf, P. K. & von Wettstein, D. (1968) *J. Lipid Res.* **9**, 425–436
- Augustinussen, E. & Madsen, M. (1965) *Physiol. Plant.* **18**, 828–837
- Bennett, J. (1977) *Nature (London)* **269**, 344–346
- Bogorad, L. (1973) in *Biochemistry of Chloroplasts* (Goodwin, T. W., ed.), vol. 2, pp. 615–631, Academic Press, London and New York
- Bradbeer, J. W. (1973) in *Biosynthesis and its Control in Plants* (Millborrow, B. V., ed.) pp. 279–302, Academic Press, London and New York
- Bradbeer, J. W. (1976) *Perspect. Exp. Biol.* **2**, 131–145
- Canaani, O. D. & Sauer, K. (1977) *Plant Physiol.* **60**, 422–429
- Castelfranco, P. A., Rich, P. M. & Beale, S. I. (1974) *Plant Physiol.* **53**, 615–618
- Champigny, M. L. & Bismuth, E. (1976) *Physiol. Plant.* **36**, 95–100
- Ellis, R. J. (1977) *Biochim. Biophys. Acta* **463**, 185–215
- Griffiths, W. T. (1975) *Biochem. J.* **152**, 623–635
- Griffiths, W. T. (1978) *Biochem. J.* **174**, 681–692
- Lendzian, K. & Bassham, J. J. (1975) *Biochim. Biophys. Acta* **396**, 260–275
- Maplestone, R. E. (1978) Ph.D. Thesis, University of Bristol
- Maplestone, R. E. & Griffiths, W. T. (1977) *Biochem. Soc. Trans.* **5**, 319–321
- Markwell, J. P., Thornber, J. P. & Boogs, R. T. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1233–1235
- McFeeters, R. F. (1975) *Plant Physiol.* **55**, 377–381
- McFeeters, R. F., Chichester, C. O. & Whitaker, J. R. (1971) *Plant Physiol.* **47**, 609–618
- O'Brien, M. J. & Pows, R. (1974) *Proc. Int. Congr. Photosynthesis 3rd* 1431–1439
- Schneider, H. A. W. (1971) *Phytochemistry* **10**, 319–321
- Smith, H. (1977) in *Regulation of Enzyme Synthesis and Activity* (Smith, H., ed.), pp. 93–128, Academic Press, London
- Steer, B. T. & Gibbs, M. (1969) *Plant Physiol.* **44**, 781–783
- Stobart, A. K. & Thomas, D. R. (1968) *Phytochemistry* **7**, 1313–1316
- Sundqvist, C. (1974) *Physiol. Plant.* **30**, 143–147
- Treffry, T. (1970) *Planta (Berlin)* **91**, 279–284
- Tremolieres, A. & Lepage, P. (1971) *Plant Physiol.* **47**, 329–334
- Wheeler, G. L. & Bittensky, M. W. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4238–4242
- Wolosuik, R. A. & Buchanan, B. B. (1977) *Nature (London)* **266**, 565–567