The Photochemical Activities and Electron Carriers of Developing Barley Leaves

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The development of photochemical activities in isolated barley plastids during illumination of dark-grown plants has been studied and compared with the behaviour of plastocyanin, cytochromes *f*, *b*-559_{LP}, *b*-563 and *b*-559_{HP} and pigments P546 (C550) and P700. Electron-transport activity dependent on Photosystem 1 and cyclic photophosphorylation dependent on *N*-methylphenazonium methosulphate (phenazine methosulphate) were very active relative to the chlorophyll content after only a few minutes of illumination of etiolated leaves, and then rapidly declined during the first few hours of greening. By contrast, Photosystem 2 activity (measured with ferricyanide as electron acceptor) and non-cyclic photophosphorylation were not detectable during the first 2½ h of greening, but then increased in total amount in parallel with chlorophyll. The behaviour of the electron carriers suggested their association with either Photosystem 1 or 2 respectively. In the first group were plastocyanin, cytochrome *f* and cytochrome *b*-563, whose concentrations in the leaf did not change during greening, and cytochrome *b*-559_{LP} whose concentration fell to one-half its original value, and in the second group were cytochrome *b*-559_{HP} and pigment P546, the concentrations of which closely followed the activities of Photosystem 2. Pigment P700 could not be detected during the first hour, during which time some other form of chlorophyll may take its place in the reaction centre of Photosystem 1. The plastids started to develop grana at about the time that Photosystem 2 activity became detectable.

An understanding of the electron-transport system of the chloroplast has been difficult to achieve, largely because of our limited ability to dissociate a complex membrane system without irreversible loss of activity. An approach that has not been fully exploited is the study of the development of the photochemical systems when etiolated plants are exposed to light; this system allows one to study the assembly of the photochemical apparatus as a natural process.

The success of this method depends on the assembly of the various components of the system being staggered. The results of Butler (1965) on fluorescence induction in greening leaves of *Phaseolus vulgaris* were the first to suggest that Photosystem 1 might become active before Photosystem 2. This possibility was confirmed with the demonstration by Gylzenholm & Whatley (1968) that cyclic photophosphorylation appeared a little earlier than non-cyclic photophosphorylation. Smith (1954) showed that etiolated barley plants form a considerable amount of chlorophyll *a* during the first 10 min of continuous illumination but develop no detectable capacity for

O₂ evolution. This result might be considered together with the observation of Davenport (1952) that plastids from leaves of etiolated barley plants contained cytochrome *f* and cytochrome *b*₆. Cytochrome *b*₆ is now known to be a mixture of two components, cytochrome *b*-559_{LP} ($E'_0 = +65$ mV, pH 7) and cytochrome *b*-563, whereas mature chloroplasts contain in addition a fourth component, namely cytochrome *b*-559_{HP} ($E'_0 = +370$ mV, pH 7) (Bendall, 1968; Bendall *et al.*, 1971). Boardman (1968) has shown that cytochrome *b*-559_{HP}, although not present in etiolated bean leaves, appears after a few hours of greening.

In the work presented here observations were made on the development of photochemical activities of etiolated barley leaves that were exposed to light for increasing lengths of time. The changes in photochemical activities of the isolated plastids were related to the changes in concentration of electron carriers and to the development of normal chloroplast structure. The components studied were cytochromes *f*, *b*-563, *b*-559_{LP} and *b*-559_{HP}, the copper protein plastocyanin, pigment P700 and the pigment known as P546 (Bendall & Sofrová, 1971) or C550 (Knaff & Arnon, 1969*a*). A short report of this work has been made (Plesničar & Bendall, 1972).

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Since our work was completed Phung Nhu Hung *et al.* (1970b) have published an account of observations on the development of some photochemical activities during the greening of etiolated barley. However, our results differ from theirs in some important respects.

Experimental

Growth of barley

Seeds of barley (*Hordeum vulgare* L.) were obtained from a local dealer. They were soaked in running water for 8 h and planted in vermiculite. Plants were grown in the greenhouse for 11 days at a minimum temperature of 18°C, with additional artificial light (14 h day) in winter and shading in summer. Etiolated barley plants were grown in the dark at 20°C for 7–8 days, depending on the length of the subsequent greening period. Plants were always 8 days old at the time of the experiment. The greening of etiolated plants was performed by exposing them for various periods of time to light of approx. 600 lx from white uorescent tubes and a tungsten filament lamp.

Preparation of plastids from barley leaves

Method A. Plastids were prepared by a method similar to that of Walker (1967) by grinding leaves with a pestle and mortar in a medium containing 0.33 M-mannitol, 10 mM-KH₂PO₄, 5 mM-NaCl, 1 mM-MgCl₂ and 0.1 % bovine serum albumin (fraction V) (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.), adjusted to pH 7.5. The homogenate was squeezed through two layers of muslin, followed by gravity filtration through eight layers of muslin. Plastids were sedimented by centrifuging for 15 min at 1800 g, washed and resuspended in the same medium. This method was used for measurement of the activities of Photosystem 1 and of Photosystem 2 stimulated by NH₄Cl and also for determination of electron carriers.

Method B. Special precautions were found necessary to obtain plastids that would carry out photophosphorylation. The following procedure was developed. Leaves were disintegrated in a medium containing 0.4 M-sucrose, 50 mM-tetrasodium pyrophosphate, 10 mM-NaCl, 1 mM-MgCl₂, 1 mM-EDTA, 0.25 % bovine serum albumin (fraction V), 0.05 % cysteine and 1 % (w/v) polyethylene glycol (mol. wt. 4000), adjusted to pH 8.0, by treatment with a Braun blender for 20 s at medium speed. The homogenate was filtered and centrifuged as described above. The plastids were washed and resuspended in a medium similar to the grinding medium except that polyethylene glycol and cysteine were omitted, Tricine[*N*-tris(hydroxymethyl)methylglycine](50 mM) was substituted for pyrophosphate and the concentra-

tion of serum albumin was increased to 1.25 %. This method was used for measurement of photophosphorylation, ADP-stimulated activity of Photosystem 2 and light-induced reactions of electron carriers.

Chlorophyll determination

The total chlorophyll of plastid preparations and leaves was determined by measurements of E_{652} on 80 % -acetone extracts and calculation as recommended by Arnon (1949). The concentrations of chlorophylls *a* and *b* in 80 % -acetone extracts were calculated from the absorbance values at 626, 645 and 663 nm with the help of the equations of Anderson & Boardman (1964), which take into account the absorbance due to protochlorophyll.

Determination of fresh weight

Photochemical activities and the contents of plastid components were referred to the fresh weight of the leaves by comparison of measurements of the absorbance due to chlorophylls at 652 nm or to carotenoids at 475 nm in the 80 % -acetone extracts of leaves and isolated plastids.

Assay of photosystems

The Photosystem 1 activity of developing plastids was determined by measurements of O₂ uptake in the presence of the couple ascorbate-*NNN'*-tetramethyl-*p*-phenylenediamine as electron donor and Methyl Viologen as an autoxidizable electron acceptor (Izawa *et al.*, 1967). The reaction mixture (5 ml) contained 50 mM-Tricine and 5 mM-potassium phosphate adjusted to pH 7.8, 1 mM-MgCl₂, 5 mM-sodium ascorbate, *NNN'*-tetramethyl-*p*-phenylenediamine, 40 µg of catalase, 50 µg of Methyl Viologen and chloroplasts containing 10–20 µg of chlorophyll.

The rate of evolution of O₂, in the presence of ferricyanide as electron acceptor, was taken as a measure of the activity of Photosystem 2. The initial rate of O₂ evolution was determined either in the presence of an uncoupler (NH₄Cl) or in the presence of ADP. The reaction mixture contained 50 mM-Tricine and 5 mM-potassium phosphate, adjusted to pH 7.8, 1 mM-MgCl₂, 1 mM-K₃Fe(CN)₆, 0.25 % bovine serum albumin, chloroplasts containing 50–200 µg of chlorophyll, and either 2.4 mM-NH₄Cl or 1 mM-ADP.

O₂ exchange was measured with an O₂ electrode (Rank Bros., Bottisham, Cambs., U.K.). The suspension was illuminated with a beam from a 500 W tungsten-lamp projector filtered through red glass (Schott, RG 610) and 15 cm of water. The intensity, measured with a Hilger-Schwarz thermopile (FT 21), was 48 mW/cm².

Measurement of photophosphorylation

Photophosphorylation was determined by measurement of ATP (Lamprecht & Trautschold, 1963), and sometimes of ADP and AMP (Adam, 1963) also, by enzymic methods. Samples (0.1 ml) were taken from suspensions of chloroplasts illuminated with red light (as above) at intervals up to 2 min and plunged immediately into 0.5 ml portions of cold 6% (w/v) perchloric acid. The solutions were neutralized with 1 M-K₂CO₃ and centrifuged; 0.5 ml samples were withdrawn for assay.

Non-cyclic photophosphorylation was measured with ferricyanide as electron acceptor in the medium described above. Cyclic photophosphorylation was carried out in the following reaction mixture (4 ml): 50 mM-Tricine, 6 mM-potassium phosphate, 10 mM-NaCl and 1 mM-MgCl₂, adjusted to pH 8.0, 0.3% bovine serum albumin, 1.25 mM-ADP, 40 µg of catalase, 0.5 µM-3-(3,4-dichlorophenyl)-1,1-dimethylurea, 7.5 mM-sodium ascorbate, 49 µM-phenazine methosulphate and chloroplasts containing 10–20 µg of chlorophyll. The system was preincubated for 6 min in the dark to allow the O₂ concentration to fall to a low value.

Estimation of electron carriers

Cytochromes (Bendall *et al.*, 1971) and plastocyanin (Plesničar & Bendall, 1970) were determined as previously described. Absorption difference spectra were measured with a sensitive split-beam spectrophotometer made in the Johnson Research Foundation, University of Pennsylvania.

The pigment P700 content of plastid preparations was determined at room temperature from the difference spectrum ascorbate (reduced) minus ferricyanide (oxidized). A difference millimolar extinction coefficient of 120 litre·mmol⁻¹·cm⁻¹ (Ke *et al.*, 1971) was used. The technique for observation of light-induced reactions at 77°K was similar to that described by Bendall & Sofrová (1971).

Electron microscopy

Centrifuged plastid pellets were fixed in 5% (w/v) glutaraldehyde containing 0.33 M-mannitol and 0.2 M-phosphate buffer, pH 7.2, for 1 h in the dark at room temperature (21°C). The pellet was then washed in water and post-fixed for 1 h at room temperature in 1% (w/v) osmic acid buffered with 17 mM-veronal acetate, pH 7.2. Subsequent preparative procedures were as described by Burgess (1970); these included uranium-lead double staining. The sections were examined in an AEI EM6B microscope.

Results

Chlorophyll formation

Fig. 1 shows the synthesis of chlorophyll *a* and *b* in 8-day-old barley plants that had been exposed to light for different lengths of time. After the initial photoconversion of protochlorophyllide present in the dark-grown leaves (Koski, 1950), there was a lag of approximately 2 h before rapid chlorophyll synthesis started. The curve for the ratio of chlorophyll *a*/chlorophyll *b* shows that the lag phase for

Fig. 1. Synthesis of chlorophyll in greening barley leaves

The curves represent the total amount of chlorophyll (●) and the ratio chlorophyll *a*/chlorophyll *b* (○). The points labelled 'green' were obtained with plants grown under normal greenhouse conditions.

chlorophyll *b* was more marked; the ratio declined from a value of 13 at 1 h of illumination to 3.6 at 6 h and then declined more gradually until it reached 2.2 in fully green leaves.

Photosystem 1 activity

Plastids isolated in darkness from etiolated barley leaves showed a low but significant activity of Photosystem 1 (Fig. 2); their chlorophyll content was too low to measure accurately and was probably no more than would be formed by photoreduction of protochlorophyll upon exposure of the preparation to the weak light of the laboratory or to the strong light required for measurement of activity. The activity on a chlorophyll basis was therefore high at this stage, but declined to a lower activity as chlorophyll synthesis ensued (Fig. 3). From this we conclude that in the early stages of greening the chlorophyll contained a greater proportion of reaction centres than in the later stages. The total activity, related to unit fresh weight of the leaves, showed a lag phase of about 2 h followed by a rapid increase until about 5 h, after which the rate of increase declined (Fig. 2).

Photosystem 1 was not saturated by the normal source of actinic light used in these experiments. When the light-intensity was varied with neutral-

density filters the reciprocal of the rate of reaction of Photosystem 1 was a linear function of the reciprocal of the light-intensity. The rate of reaction at light saturation could thus be found by extrapolation to infinite light intensity; the rates recorded in Figs. 2 and 3 are the extrapolated rates. Saturation was more difficult to achieve at the early stages of greening than later (Fig. 4), indicating that at the early stages there is a smaller proportion of accessory (or 'light-harvesting') chlorophyll. This is consistent with the conclusion reached above that the number of active centres is greater in the early stages of greening.

Photosystem 2 activity

O₂ evolution with ferricyanide as electron acceptor was coupled to phosphorylation. The photosynthetic control ratio (West & Wiskich, 1968) of barley chloroplasts was 2.3 at pH 8. Stimulated rates of O₂ evolution were measured in the presence of either NH₄Cl or ADP. In contrast with Photosystem 1, no O₂ evolution could be detected with plastids from etiolated leaves. Plastids isolated by method A showed no activity in the presence of NH₄Cl for the first 2½ h of greening and thereafter activity per g fresh wt. of leaf increased linearly with time (Fig. 2). Plastids isolated by method B showed significant activity in

Fig. 2. Activities of Photosystems 1 (○) and 2 (●) on a fresh-weight basis

For Photosystem 1, the rate of O₂ uptake was measured with the couple ascorbate-*NNN'*-tetramethyl-*p*-phenylenediamine as electron donor and Methyl Viologen as an autoxidizable electron acceptor; the rates were extrapolated to saturating light-intensity by use of the data in Fig. 4. For Photosystem 2, the rate of O₂ evolution was measured in the presence of ADP and ferricyanide as electron acceptor.

Fig. 3. Activities of Photosystems 1 (○) and 2 (●) on a chlorophyll basis

Activities were measured as for Fig. 2, except that the activity of Photosystem 2 was measured in the presence of 2.4 mM- NH_4Cl as uncoupler in place of ADP. The points labelled 'green' were obtained with plants grown under normal greenhouse conditions.

Fig. 4. Percentage saturation of Photosystem 1 achieved with red actinic light at 48 mW/cm^2

The percentage saturation was determined as described in the Results section. The point labelled 'green' was obtained with plants grown under normal greenhouse conditions.

Fig. 5. Rates of cyclic (○) and non-cyclic (●) photophosphorylation

The catalyst for cyclic phosphorylation was phenazine methosulphate and the electron acceptor for non-cyclic phosphorylation was ferricyanide (see the Experimental section for details). The points labelled 'green' were obtained with plants grown under normal greenhouse conditions.

the presence of ADP at 2½ h. The ADP-stimulated rate, on a chlorophyll basis, increased sharply to a maximum at 6 h and then declined slowly on further greening (Fig. 3). The NH_4Cl -stimulated rate was significantly lower than the rate with ADP at the early stages and only became greater after about 14 h. This discrepancy seems to be partly due to the fact that higher concentrations of NH_4Cl were required to uncouple partially green plastids than were needed in the case of normal chloroplasts; the concentration used was adequate to uncouple the latter but not the former.

Photophosphorylation

Activity of Photosystems 1 and 2 seemed at all times to be associated with phosphorylation. Cyclic photophosphorylation, with phenazine methosulphate as cofactor, behaved like Photosystem 1: the activities were very high after only brief illumination of the leaves, but declined to give a rate of about $1000 \mu\text{mol}$ of ATP/h per mg of chlorophyll at 5 h and then remained constant (Fig. 5). This rate of

Table 1. Content of electron carriers in barley plastids isolated from leaves grown in darkness or light

	Etiolated (nmol/g fresh wt.)	Light-grown	
		(nmol/g of chlorophyll)	(nmol/g fresh wt.)
Cytochrome <i>b</i> -559 _{HP}	0	2.5	2
Cytochrome <i>b</i> -559 _{LP}	5.7	2.5	2.1
Cytochrome <i>b</i> -563	4.8	4.3	3.6
Cytochrome <i>f</i>	1.9	1.8	1.5
Plastocyanin	2.8	2.5	5.2

Fig. 6. Concentration of plastocyanin during greening, measured on a fresh-weight (●) or chlorophyll (▲) basis

For details see the text.

photophosphorylation compares favourably with that reported by Hill & Walker (1959) for *Chenopodium bonus-henricus* and by Avron (1960) for Swiss-chard chloroplasts.

The curve for non-cyclic photophosphorylation with ferricyanide was similar to that for Photosystem 2. No phosphorylation could be detected until the leaves had been greened for about 2½ h and the curve then rose steeply to a maximum rate of about 700 μmol of ATP/h per mg of chlorophyll at 4 h and declined to about 250 μmol of ATP/h per mg of chlorophyll in normal green chloroplasts. The values for the $P/2e^-$ ratio varied from 1.1 to 2.0, the higher values being characteristic of the earlier stages of greening; thus both sites of phosphorylation became active together.

Electron carriers

A comparison of the concentrations of the four cytochromes and plastocyanin in etiolated and fully

green leaves is made in Table 1. Cytochrome *f*, cytochrome *b*-563, plastocyanin and cytochrome *b*-559_{LP} were all present in the etiolated leaf and the concentrations of the first three showed little or no change during greening. The greening curves for plastocyanin are shown in Fig. 6. On a fresh-weight basis there was no change in the concentration of plastocyanin but on a chlorophyll basis there was a rapid fall from an initially high value during the first 5 h of greening. The curves for cytochromes *f* and *b*-563 were very similar to those shown for plastocyanin. The concentration of cytochrome *b*-559_{LP} fell during greening to a value about one-half of that of etiolated leaf (Table 1), but otherwise it behaved much like plastocyanin (Fig. 7).

The four components mentioned above fall into a group whose qualitative behaviour during greening was similar to that of Photosystem 1. By contrast, cytochrome *b*-559_{HP} could not be detected in etiolated nor in plastids during the first 2 h of greening; its synthesis then proceeded in parallel with that of

EXPLANATION OF PLATE I

Electron micrographs of thin sections of barley plastids isolated at various stages of greening

(a) Etioplasts, isolated and fixed in the dark, magnification $\times 3000$; (b), plastids from leaves greened for 2h, magnification $\times 31500$; (c), plastids from leaves greened for 6h, magnification $\times 31500$.

Fig. 7. Concentration of cytochromes (a) $b-559_{LP}$ and (b) $b-559_{HP}$ on a chlorophyll basis

For details see the text. The points labelled 'green' were obtained with plants grown under normal greenhouse conditions.

chlorophyll (Fig. 7). This behaviour was closely similar to that of Photosystem 2 (Fig. 3) and non-cyclic phosphorylation (Fig. 5).

Electron carriers associated with a primary photochemical reaction

Light-induced absorbance changes in plastid suspensions at 77°K revealed cytochrome $b-559_{HP}$ and pigments P700 and P546. The photoactivated oxidation of cytochrome $b-559_{HP}$ and reduction of pigment P546 could first be observed in the spectra of plastids greened for 2h (Fig. 8). The amounts of oxidation and reduction of the two components were proportional to the chlorophyll concentration during chloroplast development.

The oxidation by far-red light of pigment P700, the reaction-centre pigment of Photosystem 1, was first observed in difference spectra after 1h of illumination (Fig. 8). The content of pigment P700, determined from the irreversible light-induced changes at 702nm, appeared to be constant after 1h of illumination when referred to the chlorophyll content. The amount of pigment P700 in barley chloroplasts was estimated from difference spectra [ascorbate (reduced) minus ferricyanide (oxidized)] of chloroplast suspensions at room temperature to be 0.9nmol/mg of chlorophyll, which is rather less than the amount of cytochrome f (Table 1).

Fig. 8. Irreversible light-induced absorbance changes measured at 77°K

For details see the text. The points labelled 'green' were obtained with plants grown under normal greenhouse conditions. (a) Pigment P700; (b) pigment 546; (c) cytochrome $b-559_{HP}$.

Ultrastructural changes during greening

A low-power electron micrograph of an isolated pellet of etioplasts (Plate 1a) showed them to be only slightly contaminated with mitochondria and other membranous material. Most of the etioplasts contained a prolamellar body with an apparently regular structure and also a fairly well-developed system of primary lamellae. After 2h of illumination the prolamellar bodies had lost their regular structure and the stroma contained numerous single thylakoids, but grana formation had not started (Plate 1b). At 6h of illumination developing grana could readily be detected (Plate 1c). The structural changes taking place during the greening of barley etioplasts have been studied in more detail by Phung Nhu Hung *et al.* (1970a).

Discussion

The results reported in the present paper show that the barley etioplast is capable of extensive photochemical activity as soon as even a trace of chlorophyll is made available by exposure to light. This includes photophosphorylation as well as electron transport, but is initially limited to processes dependent on Photosystem 1. The capacity for steady O_2 evolution depends on the synthesis of components other than chlorophyll, and these do not appear until the etiolated leaves have been exposed to light for about 2½h. The time-course of greening seems to be much the same whether the plants are exposed to 60lx (Nadler *et al.*, 1972), to 600lx (as used here), or to 8000lx (Phung Nhu Hung *et al.*, 1970a).

The patterns of behaviour during greening clearly place the electron carriers that we have studied into

two main groups: on the one hand, plastocyanin and the three cytochromes *b-559_{LP}*, *b-563* and *f* are associated with Photosystem 1 and phenazine methosulphate-dependent cyclic photophosphorylation; on the other, cytochrome *b-559_{HP}* and pigment P546 are associated with Photosystem 2 and non-cyclic photophosphorylation. These conclusions are consistent with most of the published evidence regarding the functions of these components except possibly in the case of the two cytochromes *b-559*, for which no clear-cut roles have yet emerged. A close functional relation between the high-potential component and Photosystem 2 has been demonstrated (Knaff & Arnon, 1969*b*, 1971; Bendall & Sofrová, 1971; Boardman *et al.*, 1971; Erixon *et al.*, 1972). However, the component is rather easily converted into a low-potential form by a variety of treatments (Wada & Arnon, 1971; Sofrová & Bendall, 1972; Cox & Bendall, 1972; Okayama & Butler, 1972), which include exposure to the uncoupling agent carbonyl cyanide 4-trifluoromethoxyphenylhydrazide (Cramer *et al.*, 1971), and in this form it seems to be available for photo-oxidation by Photosystem 1 (Cramer *et al.*, 1971). The behaviour of the low-potential component of normal chloroplasts has not usually been clearly distinguished from that of the high-potential component or its modified form, but it does not appear to be closely associated with Photosystem 2 because its photoreduction is sensitive to ioxylnil (4-hydroxy-3,5-di-iodobenzonitrile) (D. S. Bendall, unpublished work), an inhibitor which acts in much the same way as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (Friend & Olsson, 1967); the greening experiments support this view. Although the associations reported here provide no positive proof of functional roles for the components they do demonstrate unequivocally that cytochrome *b-559_{HP}* and pigment P546 can play no essential part in phenazine methosulphate-catalysed cyclic photophosphorylation.

Pigment P700 is atypical in its behaviour. None was detected during the first hour of greening but nevertheless there was a high activity of Photosystem 1 on a chlorophyll basis during this period. This suggests that pigment P700 is not the only possible reaction-centre pigment for Photosystem 1 (cf. Rurainski *et al.*, 1971). A lag in the appearance of pigment P700 during greening of etiolated maize leaves was noted by Faludi-Dániel *et al.* (1970).

In barley, plastocyanin, cytochrome *f* and cytochromes *b-559_{LP}* and *b-563* are already present in the etiolated leaf in high concentration. This is not true for all plants, as Whatley *et al.* (1972) have shown a substantial increase in the amounts of all cytochromes during greening of *Phaseolus* leaves. However, in greening barley the rate-limiting factor for Photosystem 1 activity seems to be the quantity of pigment, both of accessory pigments, lack of which

makes light saturation difficult to achieve (Fig. 4), and of reaction centres. This implies that only a proportion of the cytochrome *f*, cytochrome *b-559_{LP}*, cytochrome *b-563* and plastocyanin is associated with a functional photosynthetic unit during the early greening period. Only a small proportion of the total cytochrome *f* can be photo-oxidized by system 1 during the first few hours (Bonner & Hill, 1963; Plesničar, 1971).

Cytochrome *b-559_{LP}* differs from the other components mentioned above in that its concentration actually falls during greening; the overall amount lost matches quite closely the amount of the high-potential component found in the green leaf. This suggests that the former might be partially converted into the latter during greening. Sofrová & Bendall (1972) have shown that the high-potential cytochrome is converted into a low-potential form by extraction of chloroplasts with heptane and can be reconstituted by addition of heptane extract or of a mixture of plastoquinone and β -carotene to the extracted chloroplasts. However, an attempt to convert some of the low-potential component of barley etioplasts into a high-potential form by addition of a heptane extract of chloroplasts was a failure.

Our results suggest that Photosystem 2 is absent during the first 2½ h of greening and that the synthesis of cytochrome *b-559_{HP}* and the pigment P546 then proceeds in parallel with the other components of the system. However, the rate-limiting factor in our assay system might be any one of the components in the chain between the water-oxidizing enzyme and the point of interaction with ferricyanide. Gyldenholm *et al.* (1971) have shown that in etiolated bean leaves no Hill-reaction activity can be detected during the first 6 h of greening, but that O₂ evolution can be detected considerably earlier in flashing light; in this case the total yield of O₂ is limited, but the yield per flash is high. Gyldenholm *et al.* (1971) concluded that the limiting factor in continuous light is a component in the chain between the two light reactions. The same might be true of barley, but this would demand that neither cytochrome *b-559_{HP}* nor pigment P546 play an essential role in O₂ evolution. Recent evidence shows that, contrary to earlier suggestions (Bendall & Sofrová, 1971; Joliot & Joliot, 1972), cytochrome *b-559_{HP}* can play no essential part (Cramer & Böhme, 1972; Cox & Bendall, 1972). Erixon & Butler (1971) have presented evidence for the identity of pigment P546 and 'Q', the primary electron acceptor of Photosystem 2, but Okayama & Butler (1972) have suggested that pigment P546 is a β -carotene-protein complex that does not itself undergo oxidation-reduction but merely responds to the redox state of the true acceptor.

The time at which grana first appeared correlated approximately with the time at which O₂ evolution was first detected. Although such a correlation has

frequently been observed (Anderson & Boardman, 1964; Gyldenholm & Whatley, 1968; Homann & Schmid, 1967; Woo *et al.*, 1970), experiments on the *Chlamydomonas* mutant ac-31 (Goodenough *et al.*, 1969; Ohad *et al.*, 1967) and some higher plants have shown that the presence of partition regions is not an absolute requirement for Photosystem 2 activity (Izawa & Good, 1966; Bishop *et al.*, 1971). Further, chloroplasts of red algae contain unadressed thylakoids (Brody & Vatter, 1959).

The rate-limiting step for photophosphorylation in greening barley seems to be the activity of the electron-transport system. This conclusion is consistent with observations of the presence of an adenosine triphosphatase in etioplasts (Lockshin *et al.*, 1971; Horak & Hill, 1972), but is inconsistent with the result obtained by Phung Nhu Hung *et al.* (1970b), who were unable to detect photophosphorylation in barley during the first 8 h of greening. However, the rates of photophosphorylation observed by Phung Nhu Hung *et al.* (1970b) were much lower than those reported here, even with chloroplasts from normal green leaves.

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References

- Adam, H. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 573–577, Verlag Chemie, Weinheim
- Anderson, J. M. & Boardman, N. K. (1964) *Aust. J. Biol. Sci.* **17**, 93–101
- Arnon, D. I. (1949) *Plant Physiol.* **24**, 1–15
- Avron, M. (1960) *Biochim. Biophys. Acta* **40**, 257–272
- Bendall, D. S. (1968) *Biochem. J.* **109**, 46P–47P
- Bendall, D. S. & Sofrová, D. (1971) *Biochim. Biophys. Acta* **234**, 371–380
- Bendall, D. S., Davenport, H. E. & Hill, R. (1971) *Methods Enzymol.* **23**, 327–344
- Bishop, D. G., Andersen, K. S. & Smillie, R. M. (1971) *Biochem. Biophys. Res. Commun.* **42**, 74–81
- Boardman, N. K. (1968) in *Comparative Biochemistry and Biophysics of Photosynthesis* (Shibata, K., Takamiya, A., Jagendorf, A. T. & Fuller, R. C., eds.), pp. 206–213, University of Tokyo Press, Tokyo
- Boardman, N. K., Anderson, J. M. & Hiller, R. G. (1971) *Biochim. Biophys. Acta* **234**, 126–136
- Bonner, W. D. & Hill, R. (1963) in *Photosynthetic Mechanisms of Green Plants* (Kok, B. & Jagendorf, A. T., eds.), pp. 82–90, National Academy of Sciences–National Research Council, Washington
- Brody, M. & Vatter, A. E. (1959) *J. Biophys. Biochem. Cytol.* **5**, 289–294
- Burgess, J. (1970) *Protoplasma* **69**, 253–264
- Butler, W. L. (1965) *Biochim. Biophys. Acta* **102**, 1–8
- Cox, R. P. & Bendall, D. S. (1972) *Biochim. Biophys. Acta* **283**, 124–135
- Cramer, W. A. & Böhme, H. (1972) *Biochim. Biophys. Acta* **256**, 358–369
- Cramer, W. A., Fan, H. N. & Böhme, H. (1971) *J. Bioenerg.* **2**, 289–303
- Davenport, H. E. (1952) *Nature (London)* **170**, 1112–1114
- Erixon, K. & Butler, W. L. (1971) *Biochim. Biophys. Acta* **234**, 381–389
- Erixon, K., Lozier, R. & Butler, W. L. (1972) *Biochim. Biophys. Acta* **267**, 375–382
- Faludi-Daniel, Á., Amesz, J. & Nagy, A. H. (1970) *Biochim. Biophys. Acta* **197**, 60–68
- Friend, J. & Olsson, R. (1967) *Nature (London)* **214**, 942–943
- Goodenough, U. W., Armstrong, J. J. & Levine, R. P. (1969) *Plant Physiol.* **44**, 1001–1012
- Gyldenholm, A. O. & Whatley, F. R. (1968) *New Phytol.* **67**, 461–468
- Gyldenholm, A. O., Palmer, J. M. & Whatley, F. R. (1971) in *Energy Transduction in Respiration and Photosynthesis* (Quagliariello, E., Papa, S. & Rossi, C. S., eds.), pp. 369–374, Adriatica Editrice, Bari
- Hill, R. & Walker, D. A. (1959) *Plant Physiol.* **34**, 240–245
- Homann, P. H. & Schmid, G. H. (1967) *Plant Physiol.* **42**, 1619–1632
- Horak, A. & Hill, R. D. (1972) *Plant Physiol.* **49**, 365–370
- Izawa, S. & Good, N. E. (1966) *Plant Physiol.* **41**, 544–552
- Izawa, S., Connolly, T. N., Winget, G. D. & Good, N. E. (1967) *Brookhaven Symp. Biol.* **19**, 169–187
- Joliot, P. & Joliot, A. (1972) in *Proceedings of the 2nd International Congress on Photosynthesis Research, Stresa* (Forti, G., Avron, M. & Melandri, A., eds.), vol. 1, pp. 26–38, Junk, The Hague
- Ke, B., Ogawa, T., Hiyama, T. & Vernon, L. P. (1971) *Biochim. Biophys. Acta* **226**, 53–62
- Knaff, D. B. & Arnon, D. I. (1969a) *Proc. Nat. Acad. Sci. U.S.* **63**, 963–969
- Knaff, D. B. & Arnon, D. I. (1969b) *Proc. Nat. Acad. Sci. U.S.* **64**, 715–722
- Knaff, D. B. & Arnon, D. I. (1971) *Biochim. Biophys. Acta* **226**, 400–408
- Koski, V. M. (1950) *Arch. Biochem.* **29**, 339–343
- Lamprecht, W. & Trautschold, I. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 543–551, Verlag Chemie, Weinheim
- Lockshin, A., Falk, R. H., Bogorad, L. & Woodcock, C. L. F. (1971) *Biochim. Biophys. Acta* **226**, 366–382
- Nadler, K. D., Herron, H. A. & Granick, S. (1972) *Plant Physiol.* **49**, 388–392
- Ohad, I., Siekevitz, P. & Palade, G. E. (1967) *J. Cell Biol.* **35**, 553–584
- Okayama, S. & Butler, W. L. (1972) *Plant Physiol.* **49**, 769–774
- Phung Nhu Hung, S., Lacourly, A. & Sarda, C. (1970a) *Z. Pflanzenphysiol.* **62**, 1–16
- Phung Nhu Hung, S., Hoarau, A. & Moyse, A. (1970b) *Z. Pflanzenphysiol.* **62**, 245–258
- Plesničar, M. (1971) Ph.D. Thesis, University of Cambridge
- Plesničar, M. & Bendall, D. S. (1970) *Biochim. Biophys. Acta* **216**, 192–199
- Plesničar, M. & Bendall, D. S. (1972) in *Proceedings of the 2nd International Congress on Photosynthesis Research, Stresa* (Forti, G., Avron, M. & Melandri, A., eds.), vol. 3, pp. 2367–2374, Junk, The Hague

- Rurainski, H. J., Randles, J. & Hoch, G. E. (1971) *FEBS Lett.* **13**, 98–100
- Smith, J. H. C. (1954) *Plant Physiol.* **29**, 143–148
- Sofrová, D. & Bendall, D. S. (1972) in *Proceedings of the 2nd International Congress on Photosynthesis Research, Stresa* (Forti, G., Avron, M. & Melandri, A., eds.), vol. 1, pp. 561–567, Junk, The Hague
- Wada, K. & Arnon, D. I. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 3064–3068
- Walker, D. A. (1967) in *Biochemistry of Chloroplasts* (Goodwin, T. W., ed.), vol. 2, pp. 53–69
- West, K. R. & Wiskich, J. T. (1968) *Biochem. J.* **109**, 527–532
- Whatley, F. R., Gregory, P., Haslett, B. G. & Bradbeer, J. W. (1972) in *Proceedings of the 2nd International Congress on Photosynthesis Research, Stresa* (Forti, G., Avron, M. & Melandri, A., eds.) vol. 3, pp. 2375–2381, Junk, The Hague
- Woo, K. C., Anderson, J. M., Boardman, N. K., Downton, W. J. S., Osmond, C. B. & Thorne, S. W. (1970) *Proc. Nat. Acad. Sci. U.S.* **67**, 18–25