STUDIES ON THE GREENING OF DARK-GROWN BEAN PLANTS

II. DEVELOPMENT OF PHOTOCHEMICAL ACTIVITY

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[*Manuscript received October* 3, 1963]

Summary

A study of the photochemical activity of plastids isolated from etiolated bean leaves which had been illuminated for increasing periods was undertaken. Proplastids isolated from etiolated leaves were inactive in the Hill reaction. Photochemical ferricyanide reduction was obtained with plastids isolated from etiolated leaves which had been illuminated for 6 hr. The reduction increased to a maximum value of 400 μmoles ferricyanide/mg chlorophyll/hr after 10 hr of illumination and thereafter decreased to 300 μmoles ferricyanide/mg chlorophyll/hr for mature bean chloroplasts. Plastids isolated from etiolated leaves illuminated for 16 hr reduced nicotinamide adenine dinucleotide phosphate (NADP) at a rate less than half that of mature chloroplasts, while 8-hr plastids showed no NADP reduction.

3-(*p*-chlorophenyl)-1,1-dimethyl urea (CMU) at a concentration sufficient to cause 100% inhibition of ferricyanide reduction by mature chloroplasts had no effect on the reduction by 6-hr plastids; as the plastid developed the level of inhibition increased.

The photochemical activity of the developing plastid is discussed in relation to its structure.

I. INTRODUCTION

Dark-grown bean leaves contain protochlorophyll which is rapidly converted to chlorophyll when the leaves are exposed to light (Smith and Benitez 1954). In Part I of this series (Boardman and Anderson 1964), it has been shown that protochlorophyll is confined to a few small centres within the proplastids of dark-grown leaves whereas, in mature chloroplasts of green leaves, chlorophyll is confined to the grana regions.

Although the development of chloroplast structure has been studied in relation to the chlorophyll changes that occur when etiolated leaves are greened (Eilam and Klein 1962), little attention has been focused on the relationship between the development of chloroplast structure and the development of photochemical activity. Smith (1954) has investigated the relationship between oxygen evolution and chlorophyll content of dark-grown barley leaves after illumination, and Smith, French, and Koski (1952) have reported a few measurements of the Hill activity of plastids isolated from such leaves. They concluded that the Hill activity developed concurrently with chlorophyll content.

The aim of the present work was to measure some photochemical activities of plastids isolated from dark-grown plants, which had been illuminated for varying periods under identical conditions with those used in the studies on structure (Boardman and Anderson 1964), and to attempt to correlate the changes in such activities, both with the development of plastid structure and changes in the chlorophyll content of the developing plastid.

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II. Methods

(a) *Plant Materials*

Bean plants (*Phaseolus vulgaris* L. cv. Brown Beauty) were grown in the dark and subsequently illuminated for varying periods at 400 f.c. as described previously (Boardman and Anderson 1964).

Mature bean chloroplasts were prepared from plants grown in flats of fertilized sand–peat moss mixture (1 : 1) in a glasshouse maintained at 25°C by day and 22°C by night. Spinach (*Spinacia oleracea* L.) was grown in water culture according to the procedure of Spencer and Possingham (1960).

(b) *Preparation of Plastids*

The leaves (5 g) were ground in a Servall Omni-Mixer at 40% of the line voltage for 30 sec in 50 ml of a phosphate buffer (0·05M, pH 7·8) containing sucrose (0·4M) and KCl (0·01M). In some experiments (see text) sodium ethylenediaminetetra-acetate (EDTA, 0·005M) was added to the buffer. The homogenate was filtered first through four layers of muslin, then through four layers of Kleenex paper tissue and the plastids sedimented by centrifugation at 1000 *g* for 10 min. The plastids sedimented over a dense pellet of starch; therefore care was taken in the resuspension to leave behind as much of the starch as possible in the tube. The resuspended plastids were diluted to 40 ml with fresh buffer and the centrifugation repeated. All operations were performed at or close to 0°C.

Chloroplasts from mature bean or spinach leaves were isolated and purified in the same manner except that the leaves were ground in the Servall Omni-Mixer for 15 sec at 35% and 10 sec at 58% of the line voltage. The plants were kept in the dark for 16–24 hr prior to the harvesting of the leaves to minimize the amount of starch.

(c) *Assay of Hill Reaction Rates*

The procedures used followed the methods of Jagendorf and Margulies (1960). The temperature during the reactions was approximately 20°C and illumination of more than saturating intensity (4500 f.c.) was provided by a 250-W Photoflood lamp.

Reduction of ferricyanide was measured in two ways:

(1) A reaction mixture containing plastids (0·040 mg chlorophyll), 3·0 μmoles ferricyanide, 140 μmoles NaCl, and 80 μmoles Tris, pH 8·2, in a volume of 6 ml was prepared, and divided between two cuvettes, one of which was kept in the dark and the other illuminated for 4 min. The difference in extinction at 420 mμ was determined.

(2) Aliquots (3 ml) of the reaction mixture given in (1) were illuminated for 4 min at room temperature in centrifuge tubes. After illumination, 0·3 ml of 20% trichloroacetic acid (TCA) was added to the illuminated and dark (control) tubes, the precipitates removed by centrifugation, and the extinction of the supernatants measured at 420 mμ (Jagendorf 1962).

In inhibition studies, 3-(*p*-chlorophenyl)-1,1-dimethyl urea (CMU) was added as 0·1 ml of a solution in 95% ethanol, and an equal volume of 95% ethanol was added to the control tubes.

The reduction of nicotinamide adenine dinucleotide phosphate (NADP) was measured by the increase in extinction at 340 mμ after successive illuminations of 2 min. The reaction mixture (3 ml) contained plastids (0·025 mg chlorophyll), 80 μmoles Tris (pH 7·8), 0·6 mg NADP, 10 μmoles $MgCl_2$, and 2 units of phosphopyridine nucleotide reductase (PPNR) purified according to method A ("extract of acetone precipitate") of San Pietro and Lang (1958).

The restoration of NADP reduction of aged plastids was attempted by the addition of 0·2 μmole 2,6-dichlorophenolindophenol (DCIP) and 10 μmoles of sodium ascorbate to the reaction mixture.

(*d*) *Determination of Chlorophylls*

The chlorophyll content of green leaves was determined by the method of Arnon (1949). The pigment concentrations of etiolated leaves and etiolated leaves illuminated for up to 12 hr were determined spectrophotometrically in 80% acetone. The leaves (2 g) were ground in 25 ml 85% acetone, the precipitate removed by centrifugation, and extinction of the supernatant measured at 700, 663, 645, and 626 mμ. The specific absorption coefficients (l/g cm) used for protochlorophyll were 0·20 at 663 mμ, 4·85 at 645 mμ, and 34·90 at 626 mμ respectively. Together with the specific absorption coefficients for chlorophyll *a* and chlorophyll *b* as determined by Mackinney (1941), the following simultaneous equations were derived:

$$E_{663\ m\mu} = 82{\cdot}04C_a + 9{\cdot}27C_b + 0{\cdot}20P,$$

$$E_{645\ m\mu} = 16{\cdot}75C_a + 45{\cdot}60C_b + 4{\cdot}85P,$$

$$E_{626\ m\mu} = 14{\cdot}90C_a + 11{\cdot}66C_b + 34{\cdot}90P.$$

where C_a, C_b, and P represent the pigment concentrations in mg/ml for chlorophyll *a*, chlorophyll *b*, and protochlorophyll, and E is the extinction. These equations may then be solved to give the individual pigment concentrations in μg/ml in terms of the extinctions at the various wavelengths:

$$C_a = 12{\cdot}67E_{663} - 2{\cdot}65E_{645} - 0{\cdot}29E_{626},$$

$$C_b = -4{\cdot}23E_{663} + 23{\cdot}60E_{645} - 0{\cdot}33E_{626},$$

$$P = -3{\cdot}99E_{663} - 6{\cdot}76E_{645} + 29{\cdot}60E_{626}.$$

III. Results

(*a*) *Hill Reaction Activity*

(i) *Reduction of Ferricyanide*

Chloroplasts isolated from freshly harvested mature bean leaves reduced ferricyanide at a rate comparable with that obtained with spinach chloroplasts (Table 1). Bean chloroplasts rapidly lost activity if allowed to stand at 0°C, and

after 4 hr were almost inactive. Spinach chloroplasts on the other hand may be stored at 0°C for as long as 1 week with only a small loss in their ability for ferricyanide reduction (Table 1). This finding is consistent with the results of Margulies and Jagendorf (1960) who found that chloroplasts isolated from bean leaves stored in the dark at 0–4°C for 1 or 2 days showed low Hill activities, but chloroplasts isolated from spinach leaves stored for 5 days still showed high Hill activity.

Addition of a bean chloroplast suspension aged for 6 hr to a spinach chloroplast suspension gave a total activity equal to the sum of individual activities, indicating that no soluble inhibitor was present in the bean chloroplast suspension. No further attempt was made to investigate this *in vitro* loss of Hill activity, and reduction was investigated immediately after isolation of the plastids.

TABLE 1

EFFECT OF AGING ON FERRICYANIDE REDUCTION BY BEAN AND SPINACH CHLOROPLASTS

Chloroplast Source	Time Chloroplasts Aged at 0°C				
	0 Hr	4 Hr	1 Day	2 Days	5 Days
	Amount of Ferricyanide Reduction (μmoles/mg chlorophyll/hr)				
Bean	325·0	136·0	0	0	0
Spinach	435·0	425·0	412·0	412·0	407·0

Proplastids isolated from etiolated leaves reduced ferricyanide in the dark, and no increase in the extent of reduction occurred in the light. Plastids isolated from leaves which had been illuminated for less than 6 hr also reduced ferricyanide in the dark. Although some photochemical reduction (excess of reduction in light over that in dark) was observed with these plastids, the results were erratic and no reliable values can be presented for the 1–5-hr period.

Plastids isolated from plants which had been illuminated for at least 6 hr did not reduce ferricyanide in the dark, but they showed considerable activity for photochemical reduction (Fig. 1). The rate of reduction increased further as the plastid developed (illumination period of 6–10 hr) to a maximum value of about 400 μmoles ferricyanide/mg chlorophyll/hr (Fig. 1). With further plastid development the reduction rate decreased to the value obtained by mature bean chloroplasts (i.e. 300 μmoles ferricyanide/mg chlorophyll/hr). As shown in Figure 1 there was reasonable agreement between the two procedures used for ferricyanide reduction.

(ii) *Effect of CMU*

This reagent is an inhibitor of the Hill reaction (Jagendorf and Margulies 1960). A CMU concentration of $6{\cdot}6 \times 10^{-5}$M caused 100% inhibition of ferricyanide reduction by mature bean chloroplasts and plastids isolated from etiolated leaves which had been illuminated for 14 hr or longer. The same concentration of CMU,

however, did not completely inhibit the ferricyanide reduction by less-developed plastids (Fig. 1). No CMU inhibition of ferricyanide reduction was obtained for 6-hr plastids, and the percentage inhibition increased as the plastid developed. Thus the rate of ferricyanide reduction was inhibited 50% with 8-hr plastids and 75% with 10-hr plastids.

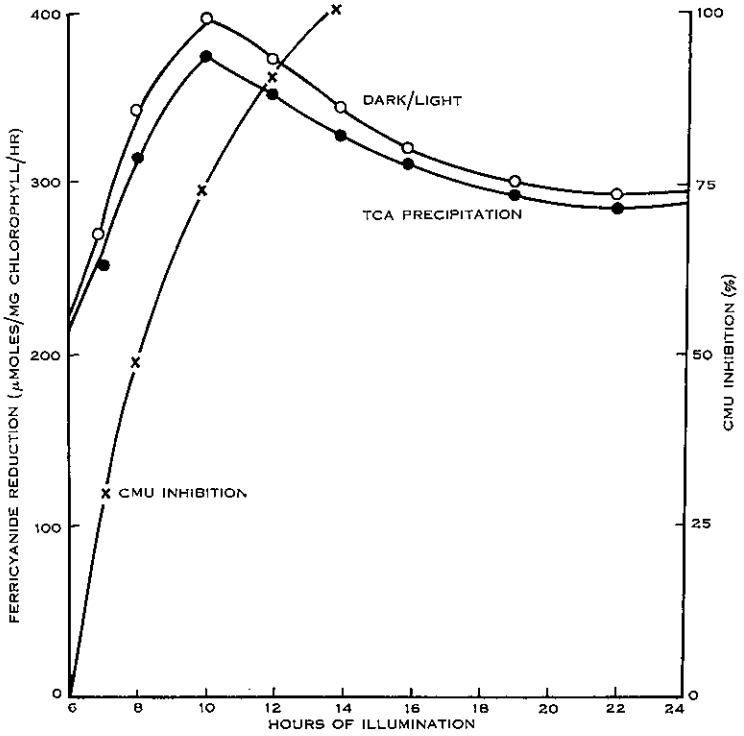

Fig. 1.—Hill reaction activity of plastids isolated from etiolated bean leaves illuminated for varying amounts of time. The ferricyanide reduction was measured in two ways (○, ●) as cited in Section II(c). × Inhibitory effect of CMU on the Hill reaction activity of plastids.

(iii) *Reduction of NADP*

The rate of reduction of NADP by spinach chloroplasts was dependent on the amount of PPNR added to the mixture. Under the assay conditions used, the concentration of PPNR was adjusted to give an NADP reduction rate by spinach chloroplasts of 57·6 μmoles/mg chlorophyll/hr. Using the same amount of enzyme, the maximum rate of NADP reduction by mature bean chloroplasts was 30–40 μmoles NADP/mg chlorophyll/hr (Table 2). These chloroplasts were prepared by grinding the leaves in a sucrose–phosphate buffer (pH 7·8) which contained no EDTA, since the reduction was slower if EDTA was included in the buffer. The mature bean chloroplasts rapidly lost activity for NADP reduction if allowed to stand at 0°C. Furthermore the activity was not restored by the addition of sodium ascorbate and DCIP to the reaction mixture, a treatment which was used by Vernon and Zaugg (1960) to restore the activity for NADP reduction of aged spinach chloroplasts.

The lower activity of bean chloroplasts is not due to the presence of a soluble inhibitor in the chloroplast suspension, since mixtures of bean and spinach chloroplasts showed an activity equal to the sum of the individual activities.

The rate of photoreduction of NADP by developing plastids was lower than that by mature chloroplasts. Plastids isolated from etiolated leaves illuminated for 16 hr reduced NADP at a rate less than half that of mature chloroplasts, while 8-hr plastids showed no NADP reduction. The addition of the ascorbate–DCIP couple had no effect on the rate of NADP reduction by the less-developed bean plastids.

TABLE 2
NADP REDUCTION BY VARIOUS PLASTID FRACTIONS

Plastid Source	NADPH Formed (μmoles/mg chlorophyll/hr)
Mature bean	30–40
Etiolated bean	
Illuminated 24 hr	20–30
Illuminated 16 hr	10–16
Illuminated 8 hr	0
Spinach	58

(*b*) *Chlorophyll Synthesis*

The formation of chlorophyll which occurred during the illumination of dark-grown bean leaves at 400 f.c. is shown in Figure 2. This curve is in general agreement with the extensive work done on chlorophyll formation during illumination (Egle 1960). Etiolated leaves contain protochlorophyll, which is rapidly converted to chlorophyll *a* when the leaves are illuminated. There is a lag phase of about 3 hr during which no new chlorophyll is formed; thereafter extensive synthesis of both chlorophyll *a* and *b* occur (Fig. 2). Between 3 and 6 hr of illumination, there is a rapid decrease in the ratio of chlorophyll *a* to chlorophyll *b*. From 16 hr of illumination onwards, the ratio is the same as that of mature leaves, i.e. 2·9.

IV. DISCUSSION

One of the aims of the present study was to look for some correlation between the development of photochemical activity and the development of plastid structure. Photochemical reduction of ferricyanide without an accompanying reduction in the dark was obtained with plastids isolated from etiolated leaves which had been illuminated for at least 6 hr and this time corresponds with the first appearance of the grana-like structures in the originally transparent regions of the isolated plastids (Boardman and Anderson 1964). The majority of the 6-hr plastids still contained the "1 μ centres"; at least 12 hr of illumination were necessary for the disappearance of all the 1 μ centres.

In the period from 6 to 12 hr, during which time most of the 1 μ centres disappear and are replaced by grana-like structures, the photochemical reduction of ferricyanide by the isolated plastids becomes increasingly sensitive to inhibition by CMU. The reduction by plastids isolated from plants illuminated for 14 hr is completely inhibited by CMU, and by this time, all the 1 μ centres have vanished and the plastids resemble mature chloroplasts, although smaller. Thus, there appears to be a good

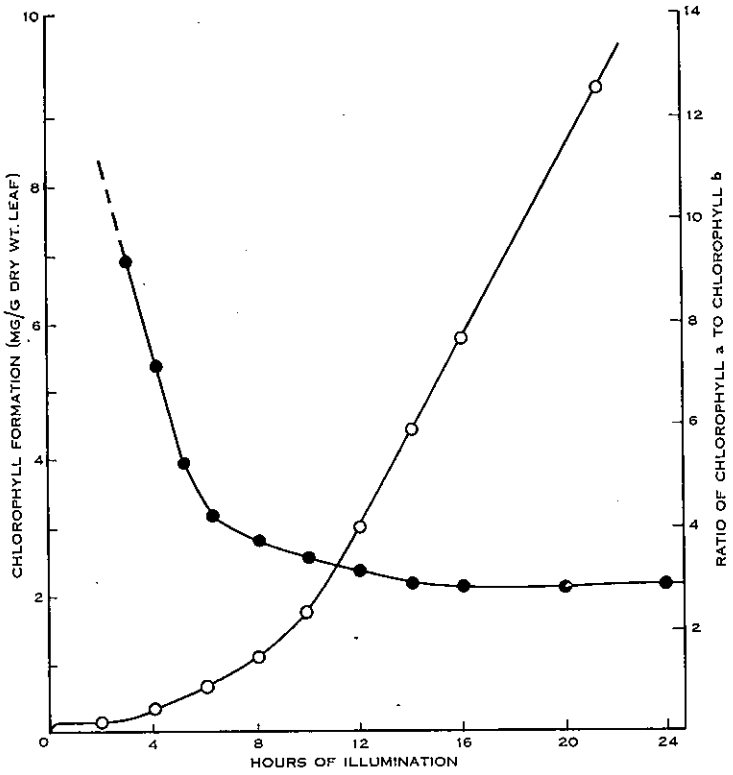

Fig. 2.—Formation of chlorophyll in etiolated bean leaves after varying amounts of illumination (400 f.c.): ○ total chlorophyll; ● ratio of chlorophyll *a* to chlorophyll *b*.

correlation between the time at which the plastids resemble mature chloroplasts as viewed in the microscope and the time at which the plastids were capable of a photochemical ferricyanide reduction typical of mature chloroplasts from green leaves.

In leaves which had received 6 hr of illumination, the total chlorophyll content had increased eightfold on the amount present in etiolated leaves which had been illuminated briefly, and the chlorophyll *a*/chlorophyll *b* ratio had declined to 4·1. Thereafter the ratio decreased only slowly and it did not reach the value of mature leaves (2·9) until an illumination time of 12–14 hr. By this time the photochemical dye reduction was completely inhibited by CMU.

CMU is a potent inhibitor of photosynthesis in green plants and algae, but the mechanism of its action is not known. However, evidence that CMU inhibits on the oxygen pathway is provided by the work of Bishop (1958), Jagendorf and Margulies (1960), and Vernon and Zaugg (1960). Bishop (1958) showed that *Scenedesmus*, which had been adapted to photoreduction under an atmosphere of hydrogen, was capable of carbon dioxide fixation in the light, but no oxygen was evolved if CMU was present. Jagendorf and Margulies (1960) and Vernon and Zaugg (1960) showed that both CMU and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) inhibit the photoreduction of NADP by isolated spinach chloroplasts; most of the activity for NADP reduction was restored by the addition of sodium ascorbate and DCIP or sodium ascorbate and *o*-chlorophenol-2,6-dichloroindophenol (TCIP).

Recent evidence suggests that in green plants and algae, there are two distinct, primary, photochemical oxidation–reduction reactions in the process of photosynthesis (Clayton 1963). These primary reactions are thought to be driven by the light absorbed by two pigment systems, system 1 and system 2. Absorption of light by system 1 results in the oxidation of a cytochrome and the reduction of NADP, while light absorbed by system 2 results in the oxidation of water and oxygen evolution and the reduction of the cytochrome (Duysens and Amesz 1962). CMU is thought to inhibit oxygen evolution by preventing system 2 from reducing the cytochrome (Duysens and Amesz 1962).

It has been shown in the present paper that in the less-developed bean plastids, there is a pathway for ferricyanide reduction which is resistant to inhibition by CMU (Fig. 1). A possible explanation for this is that the ferricyanide reduction which is not inhibited by CMU is no longer related stoichiometrically to oxygen evolution, and that the electrons for the reduction are derived not from water by means of system 2 as in mature chloroplasts, but rather from some endogenous electron donor present in the less-developed plastids. It is not known whether the pathway for electron flow which is resistant to CMU is operative *in vivo*, or whether it is an artefact of the procedures used in the isolation of the plastids.

One of the difficulties encountered in studying the development of photochemical activity in bean plastids was the rapid loss in activity of plastids stored at 0°C. Spinach chloroplasts did not show this rapid loss in activity *in vitro* (Table 1). Unfortunately etiolated spinach leaves are extremely small and it is not practical to use spinach plants for the study of development of photochemical activity.

The high starch content of bean plastids was a further disadvantage. Proplastids of etiolated leaves contain much starch; as the plastid develops the amount decreases yet some remains in 24-hr plastids. The starch granules are denser than the bulk of the plastid and tend to be torn from plastids even at the relatively low centrifugal forces used for sedimentation. This results in low yields of intact plastids.

The unicellular alga *Euglena gracilis* may well be more suitable for studies of the development of photochemical activity. Although *Euglena* cells contain storage polysaccharide granules, these are located outside the chloroplasts. Dark-grown *Euglena* cells contain proplastids with fluorescing centres (Gibor and Granick 1962) very similar to those reported for proplastids from dark-grown bean leaves (Boardman and Anderson 1964).

V. Acknowledgments

The authors are indebted to Dr. D. Spencer for advice and encouragement, and the technical assistance of Mrs. Sophie Sapiets is gratefully acknowledged.

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