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The Formation of Ribulose Diphosphate Carboxylase Protein During Chloroplast Development in Barley[1]

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Abstract. **Ribulose 1,5-diphosphate carboxylase is synthesized in barley leaves growing in the dark. Upon illumination there is a marked increase in the rate of synthesis of the enzyme. The specific activity of the enzyme expressed as cpm incorporated into phosphoglyceric acid per μg of fraction I protein, after isolation shows no change either during dark growth or greening. During early stages of illumination of 7 day dark grown leaves with 320 foot-candles the enzymic activity in the water soluble protein fraction of the leaf shows a short term decline after 15 min which lasts for 30 min. Leaves greening at 2 foot-candles show a similar decline which is shifted to a time between the fourth and eighth hr after the onset of illumination.**

The increase in protein content, when dark-grown leaves are illuminated, has been studied by De Deken Grenson (4) in Chicory and by Mego and Jagendorf (13) in *Phaseolus vulgaris*. During illumination in Chicory the protein content of whole leaves increased by 60 % while that of plastids increased by 210 %. In *Phaseolus vulgaris* plastids the protein content increased by 110 % during illumination. Wildman and Bonner (19) have shown that more than 50 % of the soluble leaf proteins constitute an electrophoretically homogenous protein designated as fraction I protein. The photosynthetic CO_2 fixing enzyme in these plants is thought to be ribulose diphosphate carboxylase (carboxydismutase) and this appears to be identical with fraction I protein (10, 17, 18). Ribulose diphosphate carboxylase is considered to be localized entirely in the chloroplasts (6). Lyttleton (9) showed that in dark grown wheat leaves the relative amount of fraction I protein increased 3-fold during 20 hr of illumination.

Biggins and Park (2) observed differences in the light and dark fixation of CO_2 of barley during greening after a net increase in chlorophyll content of the leaves was detected. The Hill reaction activity of greening barley leaves is closely related to their chlorophyll content (15). Thus it seemed worthwhile to study the formation of ribulose diphosphate carboxylase during chloroplast development.

Methods and Materials

Tissue and Extraction of Soluble Proteins. Seeds of barley *Hordeum vulgare* L. cultivar Svalof's Bonus were germinated in complete darkness in trays containing moist vermiculite at 23° at a relative humidity of 70 to 80 %. The trays were watered daily with tap water. Dark-grown seedlings of various ages were illuminated with white fluorescent tubes either at 320 ft-c or 2 ft-c. The analyses were carried out on the terminal 50 mm of the primary leaves (*cf.* 7). One hundred leaf pieces were harvested, weighed and cut cross-wise into 3 to 4 mm segments and placed in 20 ml of ice cold grinding medium. The grinding medium contained 0.015 M tris; 0.002 M $MgCl_2$ and 0.0005 M cysteine hydrochloride. The pH was adjusted to 8.0 with HCl. The tissue was homogenized in the cold in a Sorval Omnimixer at its top speed for 3 min. The homogenate was squeezed through a nylon cloth of 400 mesh and centrifuged at 129,000*g* for 120 min at 4° in a Spinco L 2 centrifuge. The clear supernatant obtained contained the soluble proteins and was used for enzyme assays and protein analyses by gel filtration.

Gel Filtration. This was performed with Sephadex G-100, which was swollen at 4° with 1 % (w/v) NaCl buffered with 0.025 M tris-HCl at pH 7.5 and allowed to stand for 7 to 8 days in the cold to achieve maximum equilibration. A K 25/45 Sephadex laboratory column (Pharmacia Ltd) was packed with swollen Sephadex and was stabilized by running the eluting medium through the column for 2 days. The composition of the eluting medium was the same as that used for swelling Sephadex. The length of the gel column was 40 cm and diameter 2.5 cm. Five ml aliquots of protein solution containing approximately 2 mg of protein were loaded onto the column and eluted using a pressure head of 20 cm and a flow rate of 30 ml per hr. The effluent liquid was scanned for UV absorption in a Uvicord (LKB Ltd) and then collected in 5 ml fractions.

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All these operations were carried out in a cold room at 4°.

Estimation of Protein. Ultraviolet absorption at 280 mμ of fractions containing trichloroacetic acid precipitable material were determined. The protein concentrations of these fractions were obtained from a standard graph made from 280 mμ absorption of different concentrations of purified bovine serum albumin. Preliminary assays of protein by the method of Lowry *et al.* (8) showed that the results obtained using Folin-Ciocalteu reagent were identical to those obtained from the UV light absorption technique.

Ribulose Diphosphate Carboxylase Assay. In preliminary experiments the pH optimum and the time curve for enzyme reaction were studied. Under the reaction conditions employed barley leaf enzyme had a pH optimum of 8.0 and the reaction proceeded linearly for about 40 min. The enzyme solution (100 μl) containing approximately 40 μg of protein was added to 1 ml of reaction mixture in a small test tube and was incubated for 20 min at 25°. The composition of reaction mixture in μmoles per ml was as follows: tris 15; $MgCl_2$ 2; cysteine 0.5; ribulose 1,5-diP 0.4; $NaH^{14}CO_3$ 0.5 (8 μc). The pH was adjusted to 8.0 with HCl. The incubations were terminated by adding 50 μl of 50 % (w/v) trichloroacetic acid. Aliquots (100 μl) were placed on Whatman glass filter paper and dried for 1 hr. The labeled phosphoglyceric acid left behind on the filter paper was counted in a Beckmann Scintillation System using 10 ml of scintillation fluid. All assays were performed in duplicate and the reproducibility was better than ± 0.1 %. The composition of the scintillation fluid was: 2,5-diphenyloxazol (PPO) 4 g, 1,4-bis [2-(4-methyl 5-phenyloxazolyl)] benzene (POPOP) 200 mg; napthalene 60 g; ethylene glycol 20 ml; methanol 100 ml; dioxane 800 ml.

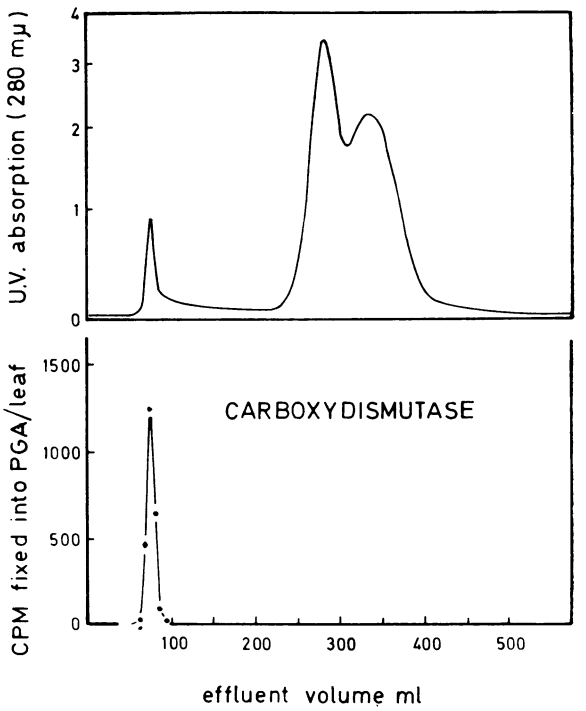

FIG. 1. The elution profile of a water soluble protein fraction of barley leaves from a Sephadex G-100 column Leaves were harvested from 7 day dark grown plants greened for 18 hr with 320 ft-c and soluble proteins prepared according to the procedure given in the text. (a) (top) Ultraviolet light absorption at 280 mμ. (b) (bottom) Ribulose diphosphate carboxylase activity. Successive 5 ml fractions were collected and assayed for enzyme activity. The reaction mixture contained in μmoles per ml: tris 15; $MgCl_2$ 2; cysteine 0.5; ribulose 1,5-diP 0.4; $NaH^{14}CO_3$ 0.5 containing 8 μc and pH adjusted to 8.0. An aliquot (500 μl) from each fraction was incubated with 1 ml of reaction mixture for 20 min at 25°.

Results

Fig. 1a shows a typical UV absorption pattern of eluates from a Sephadex G-100 column of soluble protein extracts from barley leaves. The first peak comprises of protein corresponding to a molecular weight of about 400,000 and is referred to as fraction I protein. Ribulose diphosphate carboxylase (carboxydismutase) activity was found exclusively associated with this peak (Fig. 1b). The fractions in the second peak included various low molecular weight proteins and are referred to as fraction II proteins. There was no trichloroacetic acid precipitable material in the fractions comprising the third peak. Preliminary analyses indicated that polyphenolic compounds are responsible for UV absorption in this peak.

When the soluble proteins of dark grown barley leaves of various ages and at different stages of illumination were analyzed by gel filtration, changes were observed in the size and shape of the peaks. During their growth in the dark, the primary leaves of barley accumulate fraction I protein. The leaves of 11 day dark grown plants contained twice as much fraction I protein as in 5 day old dark grown leaves. Fig. 2 summarizes the changes observed in the quantity of protein associated with the fraction I peak. During illumination for 24 hr with 320 ft-c, 5 and 7 day dark grown plants showed a 3 to 4 fold linear increase in fraction I protein. The increase of this protein in the case of 9 and 11 day dark grown plants leveled off after the first 10 hr of illumination.

Fig. 3 shows the ribulose diphosphate carboxylase activity in the soluble protein extracts from barley leaves at various stages during dark growth and greening under 320 ft-c. The changes in the activity of the enzyme in the soluble protein fraction of the leaf paralleled those observed for fraction I protein. Furthermore the specific activity of the enzyme expressed as cpm incorporated into glycerate 3-P (PGA) per μg of fraction I protein was prac-

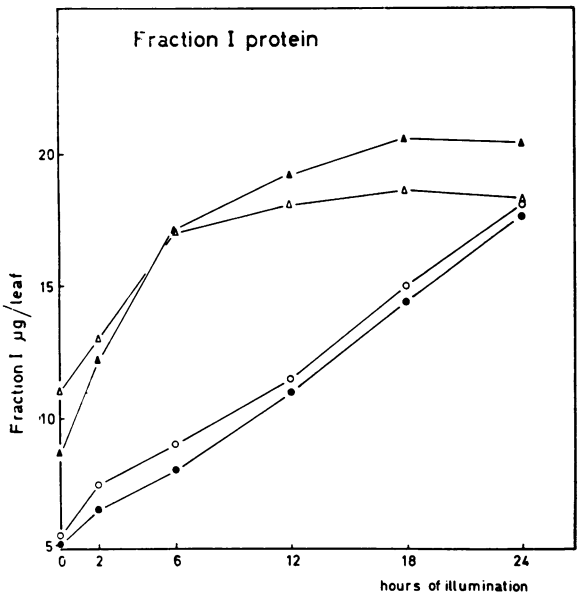

FIG. 2. Fraction I protein content in dark grown barley leaves of various ages and at different stages of illumination with 320 ft-c. —●— 5 day; —○— 7 day; —▲— 9 day; —△— 11 day.

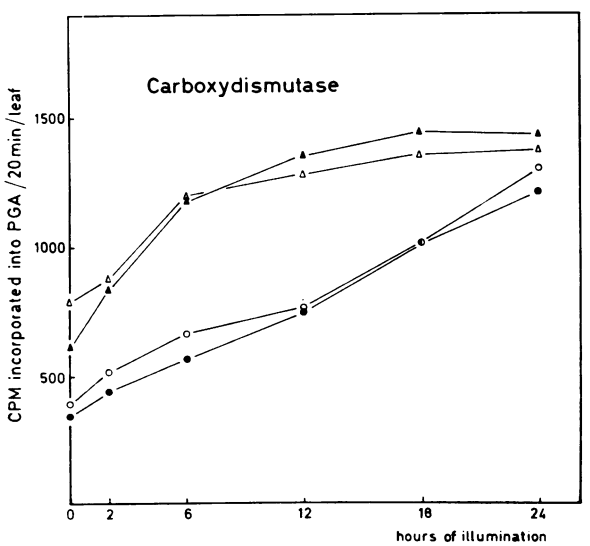

FIG. 3. The ribulose diphosphate carboxylase activity in the water soluble protein fraction of dark grown barley leaves of various ages and at different stages of illumination with 320 ft-c. One hundred µl of protein extract containing approximately 40 µg of protein was added to 1 ml of reaction mixture and incubated for 20 min at 25°. One ml of reaction mixture contained the following in µmoles: tris 15; $MgCl_2$ 2; cyteine 0.5; ribulose 1,5-diP 0.4; $NaH^{14}CO_3$ 0.5 containing 8 µc and pH adjusted to 8.0. —●— 5 day, —○— 7 day, —▲— 9 day, —△— 11 day.

Table I. *The Specific Activity of Ribulose 1,5-diP Carboxylase Isolated From Leaves of Barley at Different Stages of Development*

Age of dark grown leaves	Time of illumination with 320 ft-c Specific activity of enzyme[1]		
days	*0 hr*	*12 hr*	*24 hr*
5	65.0	68.4	69.1
7	70.7	66.8	72.5
9	71.1	70.5	70.4
11	71.8	72.0	75.5

[1] Specific activity = cpm incorporated into glycerate 3-P per 20 min per leaf / µg of fraction I protein per leaf

tically the same at all points tested (table I). This suggests that the major part of the protein comprising the fraction I peak possesses ribulose diphosphate carboxylase activity and that the changes indicated in Fig. 1 and 2 involve a synthesis of this protein.

The fraction I protein content and the ribulose diphosphate carboxylase activity in 7 day dark grown leaves at various stages of illumination with 2 ft-c is given in Fig. 4. It is of interest to note that under dim light conditions both fraction I protein content and ribulose diphosphate carboxylase activity show an increase during the first 4 hr of exposure followed by a decline lasting from the fourth to the eighth hr of illumination. A new increase of enzymic activity and fraction I protein content occurs between 8 and 24 hr of illumination. The rate of increase between 0 and 4 hr is similar to that between 8 and 24 hr. Because of these findings ribulose diphosphate carboxylase activity during early greening stages under high light intensity (320 ft-c)

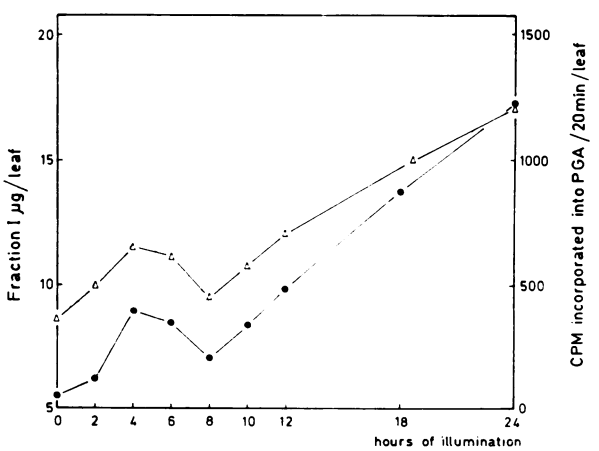

FIG. 4. The changes of ribulose 1,5-diP carboxylase activity (—△—) in the water soluble protein fraction and the content of fraction I protein (—●—) in the leaves of 7 day dark grown barley illuminated with 2 ft-c. Enzyme assay as for Fig. 3.

was reinvestigated. Measurements of the activity during the first 90 min of illumination revealed a similar pattern as found in the dim light experiment (Fig. 5).

Ribulose diphosphate carboxylase activity in the soluble protein fraction of the leaf increased rapidly during the first 15 min of illumination with 320 ft-c. This was followed by a decrease in the next 30 min and a new rise in the enzymic activity on further illumination of leaves. The soluble protein extracts from leaves illuminated for 45 min contain less ribulose diphosphate carboxylase than that from non-illuminated leaves.

Fraction I protein could be either synthesized from amino acids or assembled from preexisting low molecular weight proteins. The changes in the content of water soluble low molecular weight proteins during chloroplast development with 320 ft-c are shown in Fig. 6. An increase in the quantity of fraction II proteins was observed in barley leaves growing in the dark up to an age of 9 days. Five and 7 day dark grown plants accumulated fraction II proteins after the onset of illumination, while 9 and 11 day dark grown plants had a lag period of 2 and 12 hr respectively before an increase was observed. The amount of low molecular weight proteins accumulated in dark grown barley leaves upon illumination exceeds that of fraction I protein.

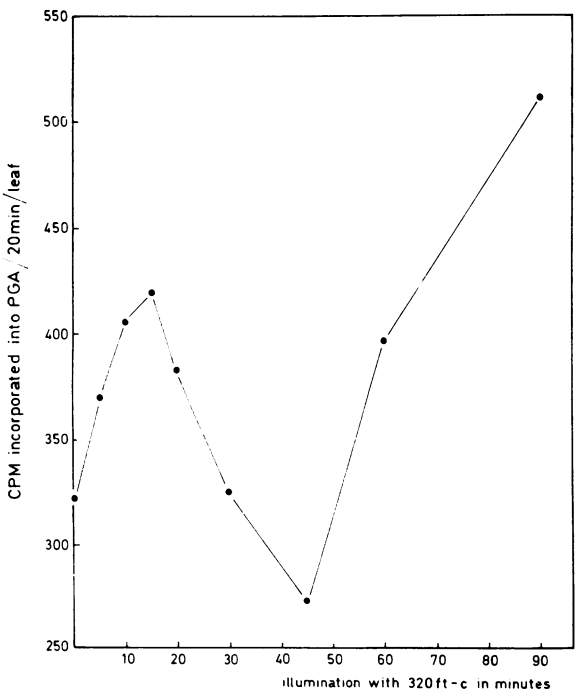

FIG. 5. The changes observed in the ribulose diphosphate carboxylase activity associated with the water soluble protein fraction of 7 day dark grown barley leaves during the first 90 min of illumination with 320 ft-c. Enzyme assay as for Fig. 3.

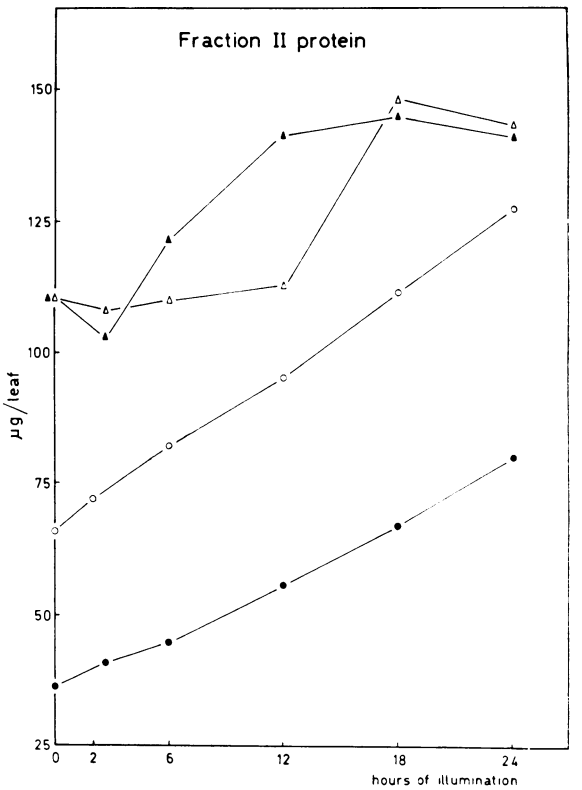

FIG. 6. The increase in fraction II proteins following illumination with 320 ft-c of dark grown barley leaves of different ages. The fraction II proteins referred to here are the proteins associated with the second UV absorption peak eluted from the Sephadex column. —●— 5 day, —○— 7 day, —▲— 9 day, —△— 11 day.

It can therefore not be decided from the data presented whether fraction I protein is assembled or synthesized *de novo*.

Discussion

The continuous synthesis of ribulose diphosphate carboxylase during dark growth of primary leaves of barley seedlings as found in this study is in agreement with earlier reports on the presence of the enzyme in dark grown bean, and wheat leaves (3, 11). Upon illumination synthesis of this enzyme takes place (3, 11, 12) and this synthesis can be inhibited by chloramphenicol (3, 5, 11, 12). Dark grown leaves are incapable of fixing $^{14}CO_2$ into glycerate 3-P (2, 16) suggesting that ribulose diphosphate does not perform its enzymic function inside dark grown leaves. The accumulation of fraction I protein during dark growth therefore raises a question whether it is simply stored or since fraction I protein and the protein moiety of protochlorophyll holochrome are considered to be identical (17) it is involved in protochlorophyllide synthesis.

At least in barley, illumination effects only an increase in the synthesis of ribulose diphosphate carboxylase which is already proceeding in the dark. After 15 min of illumination with 320 ft-c, reduction of carboxylase in the soluble protein fraction sets in, which lasts about 30 min (Fig. 5). This drop is shifted to a time between 4 and 8 hr, if low light intensity of 2 ft-c is used (Fig. 4). The decline of the enzyme from the soluble protein fraction could imply a short-term rapid break-down or more likely an incorporation of the protein into insoluble material such as chloroplast membranes. The amount of the carboxylase protein that conceivably is built into insoluble fraction can be estimated to about 10 to 20 % of the total carboxylase. According to Moudrianakis *et al.* (14) a few percent of the carboxylase activity cannot be removed from spinach chloroplast membranes by washing with water but is removed after treatment with EDTA solution. It will be of interest to study whether the carboxylase lost from soluble protein fraction during chloroplast development can be recovered from the membrane fraction with EDTA.

Between 25 and 50 % of the ribulose diphosphate carboxylase found in the stroma of the full grown chloroplasts is present in the plastids of dark grown leaves. The enzyme from dark grown leaves shows the same specific activity *in vitro* as protein isolated from the chloroplasts actively engaged in CO_2 fixation. However CO_2 fixation in the greened leaf is at least 200 times more efficient than the fixation by the isolated enzyme, suggesting that ribulose diphosphate carboxylase requires an association with a structural component of the chloroplasts such as membranes for maximum efficiency (*cf.* 1).

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