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Immunological Determination of Phosphoenolpyruvate Carboxylase and the Large and Small Subunits of Ribulose 1,5-Bisphosphate Carboxylase in Leaves of the C_4 Plant Pearl Millet[1]

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ABSTRACT

The light-dependent development of the photosynthetic apparatus in the first leaf of the C_4 plant pearl millet (*Pennisetum americanum*) was monitored by immunologically determining the concentration of phosphoenolpyruvate carboxylase and ribulose 1,5-bisphosphate carboxylase. A competitive enzyme-linked immunosorbent assay procedure using antibodies to the monomeric subunit of phosphoenolpyruvate carboxylase and the large and small subunit of ribulose 1,5-bisphosphate carboxylase was used to quantitate the amounts of these polypeptides in the first leaf of etiolated seedlings and etiolated seedlings exposed to light for varying periods of time. Phosphoenolpyruvate carboxylase was present in etiolated tissue; however, light stimulated its synthesis nearly 23-fold. Maximum accumulation of phosphoenolpyruvate carboxylase occurred approximately 4 days after etiolated plants were placed in the light. Both the large subunit and the small subunit of ribulose 1,5-bisphosphate carboxylase were present in leaves of etiolated seedlings. Light also stimulated the synthesis of both of these polypeptides, but at different rates. In etiolated leaves there was approximately a 3-fold molar excess of the small subunit to large subunit. Exposure of the etiolated leaves to light resulted in the molar ratio of the large subunit to the small subunit increasing to approximately 0.72. These data indicate that the net synthesis of these two polypeptides is not coordinately regulated at all times.

The effect of light on the photomorphogenesis of plants is well documented (4). Numerous morphological and biochemical studies indicate that the control of the expression of genes involved in the development of a fully photosynthetically competent plant may be at the level of transcription, translation, and/or enzymic activation (5, 19, 22). As most of the photomorphogenic events in plants are directed towards the establishment of the photosynthetic apparatus, it may be appropriate to document these events by measuring the appearance of certain gene products associated with the process of photosynthesis.

Since there are several types of photosynthetic systems (*i.e.* C_3, C_4, and CAM), the events surrounding the development of these different systems need to be considered individually. In the C_4 plant maize, the appearance of PEPCase[5] and RuBPCase enzymic activity has been used to monitor the biosynthetic response of an etiolated plant to light (10). Both PEPCase and RuBPCase activity increase substantially when etiolated plants are placed in the light. More recently, Nelson *et al.* (12) measured by immunodetection on protein blots the relative amounts of PEPCase and RuBPCase in maize leaves during leaf development. A more significant question is how does the mass of the large and small subunits of RuBPCase change as a result of leaves of etiolated plants turning green? The actual net accumulation of these different polypeptides can be measured during this developmental process by quantitative immunological assays (9).

We have chosen to make such measurements in the first leaf of the C_4 plant pearl millet (*Pennisetum americanum*) (8) using polyclonal antibodies to the monomeric subunit of PEPCase and the large and small subunit of RuBPCase in a competitive ELISA (2, 9, 16). This information should permit one to determine the length of time required for the light-dependent photomorphogenic development of a leaf of a C_4 plant.

MATERIALS AND METHODS

Growth of Plant Material. Pearl millet (*P. americanum*, Tift 23DB) seed (6) was planted in flats of Perlite, germinated, and kept in the dark at 25°C for varying periods of time. Etiolated plants that were to undergo greening were kept in the dark until the first leaf was completely unfurled (4 d) and then exposed to constant fluorescent light (2×10^4 ergs/cm^2·s) for varying periods of time of up to 4 d. The primary leaves of plants grown in the dark were harvested in green light. Immediately after harvesting, the plant material was frozen at −80°C.

Isolation of Proteins from Leaves. Leaves (1.0 g fresh weight) were ground in a mortar and pestle with sand (0.2 g) and 3 ml of Homogenization Buffer (50 mM Tris-HCl [pH 8.0], 1% [v/v] 2-mercaptoethanol, and 2% [w/v] SDS) at 4°C. The samples were centrifuged at 37,000*g* for 15 min. The supernatant was removed and the proteins were precipitated with 12 volumes of cold acetone at −20°C for 12 to 16 h. The protein precipitates were collected by centrifugation at 5,600*g* for 15 min, washed with 70% acetone, and dried *in vacuo*. The precipitate was

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[5] Abbreviations: PEPCase, phosphoenolpyruvate carboxylase; RuBPCase, ribulose 1,5-bisphosphate carboxylase; ELISA, enzyme-linked immunosorbent assay.

dissolved in 1 ml of 0.1 N NaOH and the samples were neutralized with 1.2 N HCl. Then 1 M Tris-HCl (pH 8.0) was added to a final concentration of 50 mM. The samples were stored at −80°C until further use.

Enzyme-Linked Immunosorbent Assay of Leaf Extracts. The quantity of monomeric subunit of PEPCase and the large and small subunit of RuBPCase in total protein of leaf extracts was determined by a competitive ELISA similar to that described by Karu and Belk (9) and modified by Bassett *et al.* (2). Rabbit antisera specific to the monomeric subunit of PEPCase and the large or small subunit of RuBPCase of pearl millet (15) was used for these experiments. Microtiter plates were rinsed with 95% ethanol and air dried. One hundred μl of freshly prepared Coating Buffer (3 mM NaN_3, 15 mM Na_2CO_3, and 35 mM $NaHCO_3$, pH 6.0) containing either 1.0 μg/ml of small subunit of RuBPCase, 1.0 μg/ml of large subunit of RuBPCase, or 1.5 μg/ml of the monomeric subunit of PEPCase was added to each well of a microtiter plate. The quantity of antigen required to saturate the wells (100 ng of small subunit of RuBPCase, 100 ng of large subunit of RuBPCase, and 150 ng of monomeric subunit of PEPCase) was determined in separate experiments by reacting a fixed quantity of antibody with increasing concentrations of antigen. The plates were incubated with Coating Buffer at 4°C for 12 to 16 h. The Coating Buffer was removed and the wells were washed three times with PBS-Tween Buffer (100 mM K-phosphate (pH 7.4), 150 mM NaCl, 3 mM NaN_3, 0.05% (v/v) Tween-20) and again air dried.

Varying amounts of protein samples from the different leaf extracts and a constant amount of either the monomeric subunit of PEPCase, the small subunit of RuBPCase, or the large subunit of RuBPCase standards were diluted into a total volume of 90 μl of PBS-Tween Buffer containing 10 μg/ml of BSA. Ten μl of an appropriate dilution of antiserum (10^{-3} of anti-PEPCase, 2×10^{-3} of anti-small subunit of RuBPCase or 10^{-2} of anti-large subunit of RuBPCase) was added to each sample. The concentration of the different antisera was chosen so that approximately 40% of the antigen bound to microtiter wells was saturated with antibodies. The samples (100 μl total volume) were incubated at 37°C and rinsed three times with PBS-Tween Buffer. Then 100 μl of goat anti-rabbit alkaline phosphatase conjugate (Sigma A-8025), diluted 10^{-3} into PBS-Tween containing 10 μg/ml BSA, was added to each well and the plates were incubated for 1 h at 37°C. The wells were washed again three times with PBS-Tween Buffer and 100 μl of substrate (1 mg/ml of *p*-nitrophenylphosphate in 10% [w/v] diethylanolamine HCl (pH 9.8), 0.4 mM $MgCl_2$, and 3 mM NaN_3) was added to each well. The samples were incubated for 30 min at room temperature and the reactions were terminated by the addition of 50 μl of 5 N NaOH. The absorbance of each sample was measured at 405 nm in a Microelisa Minireader MR590 (Dynatech, Inc.). The data was transformed using the ln-logit weighting procedures described by Rodbard and Hutt (16).

Protein and Chl Determinations. Protein concentrations of leaf extracts were determined using the Bio-Rad protein assay. Chl determinations were carried out on a specific quantity of leaves using the procedure described by Wintermans and deMots (23).

RESULTS

Growth and Development of Plant Material. Flats of seeds of pearl millet were germinated in complete darkness at 25°C. Approximately 2 d were required for germination of the seed so that the primary leaf was sufficiently large to harvest. The primary leaf was harvested from plants that had been germinated and kept in the dark for 2, 3, 4, 5, 6, 7, and 8 d. To characterize the effects of light upon the development of the photosynthetic apparatus in the plant, some of the plants that had been germinated and kept in the dark for 4 d were transferred to and kept in constant light for varying periods of time. Primary leaf samples of these plants were taken 12, 18, and 24 h and 2, 3, and 4 d after the plants were transferred into the light.

The growth and development of the primary leaf during the germination of pearl millet was monitored by measuring the total protein content per g of fresh weight of leaves (Fig. 1A). The Chl content per g of fresh leaf weight was also measured. This information was combined with the total protein content per g of fresh weight of leaves and is shown in Figure 1B as mg Chl per mg total leaf protein. When plants were germinated and kept in the dark for 8 d, there was initially a 3-fold decrease in total protein per g of fresh weight in the etiolated leaves. Then between the 5th and 6th d after planting, the total protein/g fresh weight of etiolated leaf material returned to a level similar to that found in etiolated leaves of seedlings 2 d old. If etiolated plants were transferred to the light after 4 d, there was nearly a 2.5-fold increase in total protein/g fresh weight of leaf tissue which remained constant for the next 3 d.

There was no detectable Chl in the first leaf of the plants that were germinated and kept in the dark. As soon as the 4-d-old etiolated plants were transferred to the light, Chl synthesis began and continued to increase in the leaves during the next 4 d (Fig. 1B).

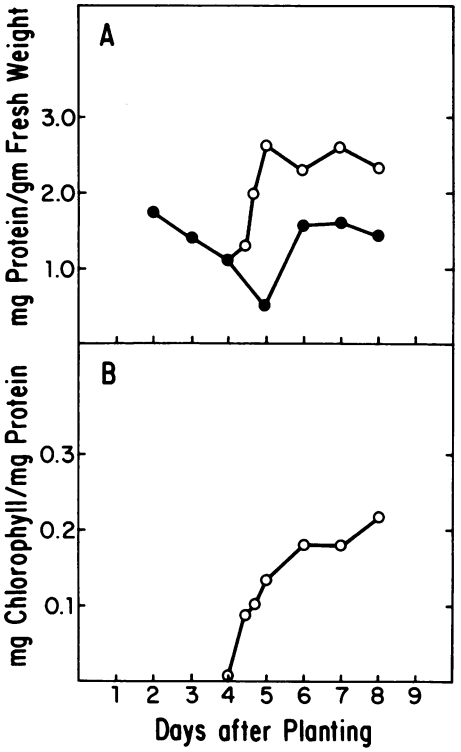

FIG. 1. Total protein and Chl content of leaves of pearl millet. Pearl millet seeds were germinated in complete darkness. The plants were kept in the dark and primary leaves were harvested 2, 3, 4, 5, 6, 7, and 8 d after planting of the seed. After 4 d of growth in the dark, some of the plants were transferred to the light and maintained in constant light. The primary leaves were collected from these plants 12, 18, 24 h and 2, 3, and 4 d after the plants had been transferred to the light. Total proteins were extracted with SDS from a given mass of fresh weight of leaf material. The total mass of protein and Chl extracted per g of fresh weight was determined. The total protein content and Chl content per given mass of fresh leaves permitted the calculation of the total Chl per total leaf protein. A, Mass of total protein per mass of fresh leaf material *versus* time after planting. B, Mass of Chl per mass of total leaf protein *versus* time after planting. (●), Leaf samples from plants grown only in the dark. (○), Leaf samples from plants grown in the dark for 4 d and transferred to the light for varying periods of time.

Characterization of the Competitive ELISA Procedure. A competitive ELISA procedure was used to determine the amount of the monomeric subunit of PEPCase and the small and large subunit of RuBPCase in proteins extracted with SDS from the primary leaf of pearl millet. Competition ELISA standard curves were generated using the monomeric subunit of PEPCase and the small and large subunit of RuBPCase. The data from these experiments was transformed using a ln-logit transformation. Figure 2 shows the standard curve generated for the large subunit of RuBPCase. Analysis of the data in this fashion permitted the determination of the minimum significant detectable amount of the antigen in question. This value is equal to the quantity of protein at logit $B/B_o = 0$ (the half maximal response). In the case of the data shown in Figure 2 for the large subunit of RuBPCase, the half maximal response was at 15 ng. Similar standard curves were generated for the small subunit of RuBPCase and the monomeric subunit of PEPCase and the half maximal responses for these two antigens were in the range of 5 and 10 ng, respectively.

Accumulation of PEPCase in Etiolated and Greening Plants. Figure 3 shows the accumulation of the monomeric subunit of PEPCase in etiolated and greening plants. The presence of the monomeric subunit of PEPCase could be detected in etiolated primary leaves as early as 2 d after planting of the seed and represented 0.12% (w/w) of the total protein. If the plants were kept in the dark, the concentration of the monomeric subunit of PEPCase remained at or near this level. When the plants were placed in the light 4 d after planting, there was a lag of approximately 24 h before PEPCase began to accumulate. The greatest net increase in PEPCase came between 1 and 2 d after the etiolated plants had been placed in the light. After the plants had been in the light for 4 d, PEPCase had increased to 3.4% (w/w) of the total leaf protein.

These results show that the synthesis of the monomeric subunit of PEPCase does not require light and that this protein is present, although in very low quantities, in plants grown solely in the dark. However, light does have a pronounced effect upon the total net accumulation of PEPCase in the primary leaf of pearl millet.

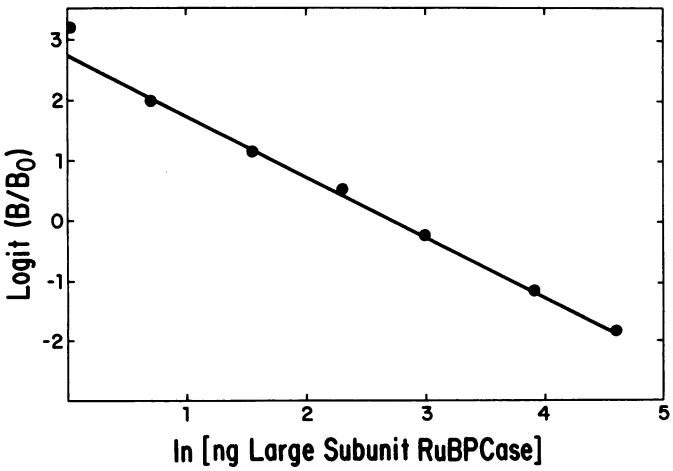

FIG. 2. Competition ELISA standard curve for the large subunit of RuBPCase. A competition ELISA curve was generated using the large subunit of RuBPCase as described in "Materials and Methods." The data were transformed using the ln-logit and weighting procedure described by Rodbard and Hutt (7). The graph is plotted such that the ordinate is expressed as ln $[(B/B_o)\ (1 - B/B_o)^{-1}]$ = logit (B/B_o), where $B = A_{405}$ (sample) − A_{405} (limiting high dose) and $B_o = A_{405}$ (limiting high dose) − A_{405} (limiting low dose) and the abscissa is the ln of the mass of large subunit of RuBPCase.

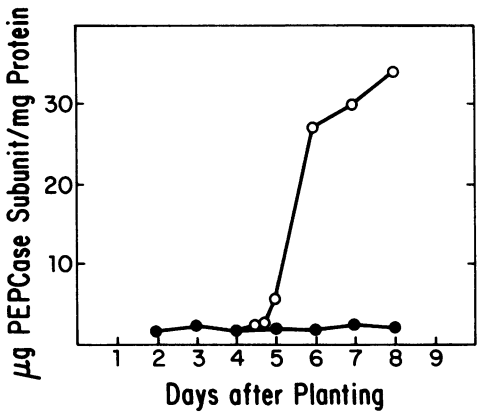

FIG. 3. Influence of light on the concentration of the monomeric subunit PEPCase in the primary leaf of pearl millet. Total protein was extracted from etiolated and greening leaves with SDS. The protein was precipitated with acetone, dissolved in NaOH, and neutralized. Total protein was determined and the mass of the monomeric subunit of PEPCase present in this protein was determined by a competitive ELISA. Mass (μg) of the monomeric subunit of PEPCase per mg of total protein in leaves of plants grown in the dark (●) and in leaves of plants grown in the dark for 4 d and then exposed to light (○).

Accumulation of the Small and the Large Subunits of RuBPCase in Etiolated and Greening Plants. Figure 4 shows the accumulation of the small and the large subunits of RuBPCase in etiolated and greening plants. Both the small and the large subunits of RuBPCase are present in the primary leaf of seedlings 2 d after planting and germinating the seeds in the dark. At this time, approximately 0.18% (w/w) and 0.21% (w/w) of the mass of the total leaf protein could be accounted for by the small and the large subunits of RuBPCase, respectively. In the next 2 d, while the plants were in the dark, the small and the large subunits increased in concentration 23- and 21-fold, respectively. When the etiolated plants were placed in the light, both the small and the large subunits again increased considerably in concentration. After the plants had been in the light for 4 d, the small subunit had increased to 5.3% (w/w) and the large subunit had increased to 10.7% (w/w) of the total leaf protein. Plants that were kept in the dark for the same period of time showed only a small increase in the quantity of small and large subunit of RuBPCase. Light obviously caused a substantial increase in quantity of both these polypeptides in the primary leaves of greening etiolated plants.

The most interesting fact regarding the quantities of the small and the large subunit of RuBPCase is that they were not found in equivalent molar quantities in plants that were germinated and maintained in the dark. If one assumes the mol wt of the mature small subunit and the large subunit to be 14 and 52 kD, respectively, one can calculate the number of mol of each of these polypeptides per mg of total leaf protein. If the number of molecules of each of these polypeptides was similar to what one finds in the holoenzyme, the molar ratio of the large subunit to small subunit would be equal to 1.0. Surprisingly, in the etiolated primary leaf the molar ratio of the large to small subunit of RuBPCase was in the range of 0.3 to 0.4. When etiolated tissue was exposed to light, this ratio increased to slightly more than 0.7. In the primary leaf of pearl millet, the molar ratio never reached the expected value of 1.0 found in the purified holoenzyme.

Since the molar ratio of the large subunit to the small subunit of RuBPCase was significantly less than 1.0 in etiolated primary leaves, there were more molecules of small subunit present than large subunit. The increase in the molar ratio of these two polypeptides when the etiolated plants were placed in the light,

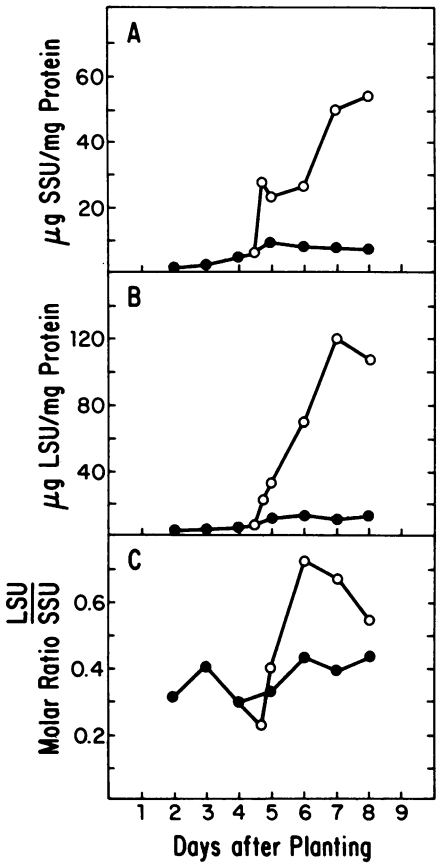

FIG. 4. Influence of light on the concentration the small subunit and the large subunit of RuBPCase in the primary leaf of pearl millet. Total protein was extracted from the etiolated and greening leaves with SDS and was prepared for analysis as described in Figure 3. The mass of the small subunit and of the large subunit of RuBPCase in this protein was determined by a competitive ELISA procedure using antibodies to these two proteins. This information is summarized as the mass (μg) of either the small subunit or the large subunit per mg of total leaf protein in the leaves. Data for plants either grown continually in the dark (●) or plants grown in the dark for 4 d and then exposed to light (○). The mass of these two proteins was converted to the number of mol of these proteins per mg of total protein and then the ratio of the mol of large subunit to small subunit was calculated. A, Mass of the small subunit of RuBPCase per mg total protein. B, Mass of large subunit of RuBPCase per mg total protein. C, Ratio of mol of large subunit to small subunit of RuBPCase.

indicated that there was a more rapid net accumulation of the large subunit than of the small subunit of RuBPCase.

DISCUSSION

The light-dependent development of the photosynthetic apparatus in the leaves of the C_4 plant maize has been monitored by following the enzymic activity of PEPCase and RuBPCase (10) and by immunochemical analysis of protein blots (12). Analysis of this developmental process in both these cases was refractile to detecting very low quantities of these enzymes and did not permit one to determine the absolute amounts of these proteins in leaf tissue. The need to determine the presence of these and other proteins in forms which are not enzymically active and/or present in very low quantities caused us to exploit the competitive ELISA. The competitive ELISA is a reliable technique which can determine the actual mass of the monomeric subunit of PEPCase and the small and large subunit of RuBPCase in a sample of protein. Quantities of all three of these polypeptides as low as 5 to 15 ng can be detected in mg quantities of total cellular protein. The validity of the competition ELISA procedure for determining the presence of these polypeptides depends upon the efficiency and reproducibility of the procedure used for preparing total leaf protein extracts. It must also be insensitive to interference by substances other than the antigen in the cell extracts that might bind to the antibody. Since we have used SDS to extract total proteins from the leaves, our results should not be affected by the possibility of any of these polypeptides being membrane bound or otherwise insoluble at some specific time during development. Nor have we observed any obvious problems of substances interfering with the binding of the antibody to the antigen. However, as in any immunoassay procedure, one must be able to use either a monospecific antibody or a pure antigen for the standard. In these experiments, we used the pure monomeric subunit of PEPCase or the small or large subunit of RuBPCase as standards.

The change in absolute quantities of the three polypeptides measured in these experiments reflects a net accumulation in mass of these proteins due to illumination of etiolated plants. The presence of all three of these proteins in etiolated tissue indicates that the genes for these polypeptides were transcribed during the germination of these seedlings in the absence of light. When the etiolated seedlings were placed in light, there was a noticeable lag of nearly 24 h before there was any significant accumulation of the monomeric subunit of PEPCase or the small or large subunits of RuBPCase. This same lag was seen in the increase of the holoenzymes PEPCase and RuBPCase as detected by enzymic activity, when etiolated maize seedlings were placed in the light (10). Since Mukerji (11) has demonstrated the presence of at least two forms of PEPCase in maize and Ting and Osmond (20, 21) have suggested the existence of at least four classes of PEPCase, it is possible that the polyclonal antibody to PEPCase is detecting different forms of PEPCase in the etiolated leaves *versus* the greening leaves. Thus, one can not actually state that light causes the increase in synthesis of that type of PEPCase found in the dark.

The most interesting observation in these studies was the significantly less than equimolar quantities of the large subunit and small subunit of RuBPCase found in the etiolated primary leaf of pearl millet. This was an unexpected result and could have only been detected by measuring the actual mass of these two polypeptides present per mg of leaf protein. The small subunit was more than 3-fold in excess in the etiolated leaf tissue. When the etiolated tissue was placed in the light, the molar ratio of these two polypeptides approached but did not reach 1.0. A similar disparity in the molar ratio of these two polypeptides has also been seen in the basal region of leaves of barley (13).

Numerous experiments have shown that the mRNAs for the small subunit and the large subunit of RuBPCase both increase when etiolated plants are placed in the light. A number of these reports argue for the coordinate induction of these two mRNAs (12, 18, 19). However, none of these studies quantitated the actual mass of the mRNAs for either of these two polypeptides or the actual quantities of these two proteins in a leaf of a plant. Sasaki *et al.* (17) and Tobin and Suttie (22) have measured the relative ratio of synthesis of these two polypeptides. Sasaki *et al.* (17) showed that in etiolated seedlings exposed to light the large subunit was synthesized more rapidly than was the small subunit of RuBPCase. Tobin and Suttie (22) also saw in *Lemna* that the rate of synthesis of the large subunit was more rapid than the rate of synthesis of the small subunit of RuBPCase. Neither of these observations are directly comparable to this work, because both Sasaki *et al.* (17) and Tobin and Suttie (22) measured the rate of synthesis of the two subunits relative to total protein synthesis rather than the net accumulation of mass of these two polypeptides per mass of protein.

Others have shown that there need not be tight coupling

between the large and subunit synthesis (1). These same researchers suggested that the synthesis of the small subunit may be entirely independent of that of the large subunit. This suggestion is supported by the fact that the small subunit can accumulate in higher plants that lack chloroplast ribosomes due to heat treatment (7). Roy *et al.* (14) showed that a major pool of *in vivo* labeled small subunit exists in pea seedlings. Since all of these studies monitored the presence or synthesis of both the large and the small subunits by radioactively labeling of these polypeptides with [^{35}S]methionine, these results can not be directly compared to our observations which show a net excess in the molar quantity of the small subunit of RuBPCase in the dark.

It is clear from this work and that of others (1, 7, 14) that there must be a different means of regulation for the expression of the small and large subunits of RuBPCase. One possible explanation for the disproportionate increase in the quantity of large subunit relative to the small subunit is that there is a significant increase in the number of genes for the large subunit during greening of etiolated leaf tissue. This occurs with an increase in the number of chloroplast DNA molecules per leaf cell during the formation of the functional photosynthetic apparatus (3). However, there may be other possible explanations for the differential expression of these two proteins. These may occur at the level of both transcription and/or translation. The development of a reasonable model for such regulation awaits the precise quantitation of the amounts of mRNAs for both the large subunit and the small subunits as well as the actual rates of synthesis of both the mRNAs and the polypeptides.

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