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Development of Ribulose 1,5-Diphosphate Carboxylase in Nonphotosynthetic Endosperms of Germinating Castor Beans[1]

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ABSTRACT

Ribulose 1,5-diphosphate (RuDP) carboxylase has been partially purified from dark-grown nonphotosynthetic endosperms of germinating castor beans (*Ricinus communis* var. Hale). The *Km* values for RuDP, HCO_3^-, and Mg^{2+} are 0.51, 33, and 1.78 mM, respectively. The pH optimum for the carboxylation reaction is pH 7.5. Germination is required for the development of the carboxylase in the endosperms. The enzyme reaches a maximal activity in 4- to 5-day-old dark-grown seedlings (which have an endosperm weight of approximately 0.75 g fresh weight/bean) and then declines. Total endosperm carboxylase activity is 1230 nmoles/min·g fresh weight which is 25 and 50% of the total activity developed in soybean and maize leaves, respectively. Specific activity of the carboxylase in crude soluble endosperm preparations (which contain enzymic and storage protein) is 0.05 μmole/min·mg protein. This is 5 times greater than the specific activity of RuDP carboxylase in soluble preparations from etiolated leaves. During germination the V_{max} of the endosperm carboxylase for RuDP increases 10-fold. Development of the enzyme is inhibited 90% by the exposure of the endosperm to 2 μg/ml cycloheximide or 50 μg/ml chloramphenicol. Light (or phytochrome Pfr) is not required for the synthesis of the enzyme. Electron photomicrographs of dark-grown endosperm cells (with peak RuDP carboxylase activity) show proplastids with several invaginations of the inner membrane but no prolamellar-like structures.

Previous reports have described the presence and properties of RuDP[2] carboxylase in castor bean endosperms (4, 15). These are the only reports of the occurrence of this enzyme in nonphotosynthetic higher plant tissue. RuDP carboxylase has been localized in the proplastids of germinating castor beans (15) and the enzyme reaches a peak activity in the dark in 4 days (4). Dark-grown etiolated leaves contain low amounts of RuDP carboxylase activity which increases substantially only after exposure of the leaves to light (6, 10, 13). The purpose of this paper was to study the unique development of RuDP carboxylase and proplastids in the endosperms of germinating castor beans.

MATERIALS AND METHODS

Plants. Castor beans (*Ricinus communis* var. Hale) were obtained from McNair Seed Company, Plainview, Texas. The seeds were dusted with Arasan. The seeds were imbibed in H_2O for 24 hr and germinated in moist vermiculite at 35 C in the dark. For developmental studies, the seedlings were harvested from 0 to 8 days. For antibiotic studies, the seedcoats were removed, and the seeds were imbibed in H_2O, 2 μg/ml CHI, or 50 μg/ml CAP. The imbibed seeds were germinated at 35 C in the dark in Petri dishes in H_2O or antibiotic solution. To study the effect of light on the development of RuDP carboxylase, the imbibed seeds, without seedcoats, were germinated in the light or dark at 35 C. The light source was General Electric cool-white 20-w fluorescent bulbs. The light intensity at the seed surface was 30 μeinsteins m^{-2} sec^{-1}.

Previously (4), it has been shown that RuDP carboxylase activity peaks in 4 day old germinating castor beans. We found that both the rate of growth of the castor bean seedlings and the rate of development of RuDP carboxylase in the seedlings are influenced by *(a)* removal of the hard seedcoat prior to germination and *(b)* the watering regime during the growth of the seedlings. Removing the seedcoats, prior to imbibing and germinating the seeds, increases the rate of seedling growth. In these seedlings growing in Petri dishes, RuDP carboxylase activity peaks 48 hr after germination. Depending on the watering regime, the peak carboxylase activity can be extended 1 to 2 days in seeds germinating in vermiculite. Because of these variables, in most experiments we have recorded the chronological age of the seedlings and the fresh weight of the endosperms. In most experiments, maximal RuDP carboxylase activity developed in seedlings with an endosperm weight between 0.60 and 0.84 g fresh weight/seed.

Enzyme Isolation and Purification. RuDP carboxylase was isolated and partially purified from endosperms of 5-day-old seedlings. (0.75 g endosperm/seed). All steps were carried out at 4 C. The endosperms were separated from the testa, hypocotyl, epicotyl, and cotyledons and rinsed with deionized H_2O. The endosperms were homogenized for 60 sec in a prechilled Waring Blendor in 0.1 M tris buffer, pH 7.5, containing 0.1 mM GSH. The homogenizing medium contained 1 ml buffer/g fresh weight of endosperm. The homogenate was filtered through two layers of cheesecloth and centrifuged 15 min at 1000*g* in a RC-5 Sorvall refrigerated centrifuge. The crude supernatant fraction was centrifuged 30 min at 17,000*g*. The soluble supernatant was fractionated with $(NH_4)_2SO_4$. The protein which precipitated between 35 and 55% of saturation with $(NH_4)_2SO_4$ was collected by centrifugation and dissolved in 3 ml of 0.1 M tris buffer, pH 7.5, containing 0.1 mM GSH. The protein solution was dialyzed overnight against 0.1 *m* tris buffer, pH 7.5, containing 0.1 mM GSH. The dialyzate was centrifuged at 17,000*g* to remove any insoluble material. The solution was layered on a Sephadex G-200 column (84 × 2.5 cm) which had been previously equilibrated with 0.1 M tris, pH 7.5, containing 0.1 mM GSH. The protein was eluted from the column with the same buffer and collected in 2-ml fractions. A plot of absorbance against fraction number shows 2 major peaks at 280 nm (Fig. 1). RuDP carbox-

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[2] Abbreviations: RuDP: ribulose-1,5-diP; PGA: 3-phosphoglyceric acid; PEP: phosphoenolpyruvate: CHI: cycloheximide; CAP: chloramphenicol.

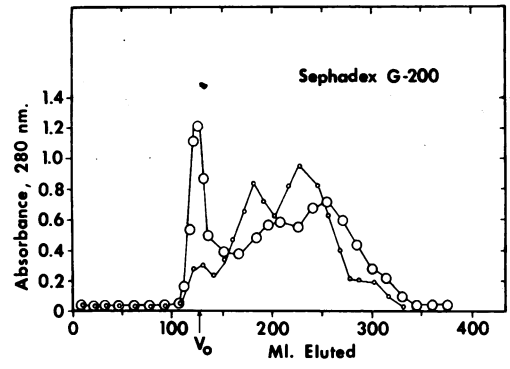

FIG. 1. Elution profile of the 35 to 55% $(NH_4)_2SO_4$ fraction on Sephadex G-200 columns. Endosperm carboxylase was eluted from the column in the void volume. O: $(NH_4)_2SO_4$ fraction from endosperms of 6-day-old germinating castor beans. o: $(NH_4)_2SO_4$ fraction from endosperms of 2-day-old germinating castor beans.

ylase was eluted from the column in the void volume. This void volume protein increases between 2-day- and 6-day-old endosperms. In these experiments a seed with maximal carboxylase activity had a fresh weight of endosperm of 0.70 to 0.80 g and was called a 6-day-old bean. The 2-day- and 4-day-old beans had endosperm fresh weights of 0.30 to 0.38 and 0.42 to 0.50 g/bean, respectively. The void volume was pooled and concentrated to 3 *A* units/ml in an Amicon Diaflo containing a UM-2 membrane. This concentrated protein solution was used as the source of RuDP carboxylase for the kinetic analysis. RuDP carboxylase from Sephadex G-200 columns was too highly adsorbed onto DEAE-cellulose columns for displacement with 0.5 M tris buffer containing 0.5 M NaCl.

Protein Determinations. Protein was determined by the method of Lowry *et al.* (12). BSA was used as the standard.

Enzyme Assay. RuDP carboxylase was assayed by a modification of the method of Benedict (4). The reaction mixture contained in μmoles: 50 tris buffer (pH 7.5); 1.25, GSH; 5, $MgCl_2$; 1.25, RuDP tetrasodium salt; 25, $KH^{14}CO_3$ which contained 5 μCi of radioactivity; 0.1 ml of enzyme and H_2O to a final volume of 0.5 ml. The reaction blank contained all of the reagents except RuDP. The reaction tubes were incubated at 35 C for 15 min. After this incubation, the tubes were placed on a N_2-bubbling apparatus and the reaction stopped with 0.1 ml of concentrated HCl. The volume in the tubes was brought to 10 ml with H_2O. The tubes were bubbled with N_2 for 15 min and the unreacted $^{14}CO_2$ was collected in a saturated solution of $Ba(OH)_2$. The reaction mixture was assayed for ^{14}C-PGA by placing a 0.1-ml aliquot in 15 ml of scintillation fluid containing 5 g of PPO, 100 g of naphthalene, 10 ml of H_2O and dioxane to 1 liter and counting to a ±0.2% error in a Beckman LS-200 B scintillation spectrometer system.

Electron Microscopy. The endosperm tissue from germinating castor beans was fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. The tissue was then exposed to a phosphate-buffered osmium tetroxide fixation, dehydrated with ethanol, and embedded in Epon. The sections were stained with uranyl acetate and lead citrate. The sections were photographed with a Hitachi HU-11E electron microscope.

RESULTS AND DISCUSSION

The results of the purification procedures for RuDP carboxylase from castor bean endosperms are shown in Table I. The enzyme has been shown to be inhibited by SO_4^{2-} (16) which accounts for the low carboxylase activity in the 35 to 55% $(NH_4)_2SO_4$ precipitate and the reappearance of activity in the 35–55% $(NH_4)_2SO_4$ dialyzate. The specific activity of RuDP carboxylase eluted from Sephadex G-200 columns was 0.200 μmole CO_2 fixed/min·mg protein. This specific activity is 10 times greater than reported by Osmond *et al.* (15) for a similar preparation of RuDP carboxylase from castor beans, but 10 times lower than more purified preparations from green leaves (7).

The specific activity of RuDP carboxylase in the soluble protein fraction from castor bean endosperms is 0.05 μmole CO_2 fixed/min·mg protein. The specific activity of RuDP carboxylase in comparable soluble protein fractions from 6-day-old dark-grown leaves is 0.011 μmole CO_2 fixed/min·mg protein (13). The enzyme activity is 5 times greater in endosperms compared to etiolated leaves.

The specific activity of RuDP carboxylase from green spinach leaves range from 0.23 to 0.35 μmole CO_2 fixed/min·mg protein (16, 20). RuDP carboxylase is a major protein in leaves and is 50% of the total soluble protein in leaf extracts (9). There is no comparable soluble protein fraction (fraction I) in germinating seeds. RuDP carboxylase from castor bean endosperms has albumin type properties (8). It is soluble in H_2O, precipitated at 55% of saturation with $(NH_4)_2SO_4$ and remains in solution following an extensive dilution by dialysis. The following composition has been reported for the protein of castor bean meal: 60% globulins, 16% albumins, 4% proteoses, and 20% glutelins (5). If the specific activity of endosperm carboxylase is corrected for the globulin content in the soluble preparation, it becomes 0.23 μmole CO_2 fixed/min·mg protein. This level of specific activity is comparable to the lower range of RuDP carboxylase reported for green leaves (16).

The pH optimum is 7.5 in tris buffer. This agrees with the value reported by Osmond *et al.* (15) but is shifted 0.5 pH unit down from the optimum found for the enzyme from green leaves (2). Another amine buffer, bis-tris, is inhibitory to the reaction above pH 6.5. Bis-tris buffer is a neutral buffer above pH 6.5 while tris buffer is protonated below 8.1. Despite both these buffers being amine buffers, the difference in their pK values may lead to an enhancement or inhibition of the enzyme activity. In addition, it is possible that bis-tris may be binding Mg^{2+} ions. Both Sugiyama *et al.* (18) and Bassham *et al.* (2) have shown that the pH optimum of RuDP carboxylase is sensitive to the concentration of Mg^{2+} ions. In agreement with the observations made by Weissbach *et al.* (19), Kuehn and McFadden (11), and Paulsen and Lane (16) phosphate buffer is inhibitory to the RuDP carboxylase reaction. Imidazole buffer also inhibits the reaction.

The *Km* value for HCO_3^- is 33 mM. High concentrations of HCO_3^- inhibit the reaction. RuDP carboxylase isolated from castor bean endosperm is similar to the high *Km* carboxylase described by Bahr and Jensen (1).

The *Km* value for Mg^{2+} is 1.78 mM. The reaction is inhibited at concentrations of Mg^{2+} greater than 20 mM and is completely inactive in the absence of Mg^{2+}.

The development of RuDP carboxylase activity in endosperms of germinating castor beans in the dark is shown in Figure 2. The enzyme activity peaks 4 days after germination and decreases to a low or negligible value 7 days after germination. There is no

Table I. *Specific Activity of Castor Bean Endosperm RuDP Carboxylase during Purification*

Enzyme Fraction	Total Protein (mg)	Specific Activity (μmoles/min·mg protein)
Crude Soluble Extract	578	0.049
35-55% $(NH_4)_2SO_4$ Pellet	123	0.001
35-55% $(NH_4)_2SO_4$ Dialyzate	123	0.069
Sephadex G-200	19.8	0.191

significant enzyme activity in dry or imbibed seeds and germination is required for the appearance of RuDP carboxylase activity in the endosperms. These data are similar to the RuDP carboxylase developmental curve previously published (4) with the important exception that total units of RuDP carboxylase/g fresh weight have now been determined. This makes possible a comparison of carboxylase units from castor bean endosperms and leaves. The RuDP carboxylase activity in endosperms is 1230 nmoles/min·g fresh weight in comparison to about 2500 nmoles/min·g fresh weight in green maize leaves, and 5000 nmoles/min·g fresh weight in green soybean leaves (6). The amount of RuDP carboxylase activity in castor bean endosperms is 25% of soybean leaves and 50% of maize leaves. The developmental curve for RuDP carboxylase is similar to the developmental curves for malate synthetase and isocitric lyase in endosperms of germinating castor beans (3).

At three different times during the development of RuDP carboxylase activity in the endosperms, the V_{max} and K_m values for RuDP were determined on the enzyme fraction eluted from Sephadex G-200 columns (Fig. 3). For enzyme preparations isolated from 2-, 4-, and 6-day-old seedlings, the V_{max} at saturating concentrations of RuDP increased 10-fold. The K_m values for 2- and 6-day-old endosperm carboxylase were 0.27 and 0.51 mM, respectively.

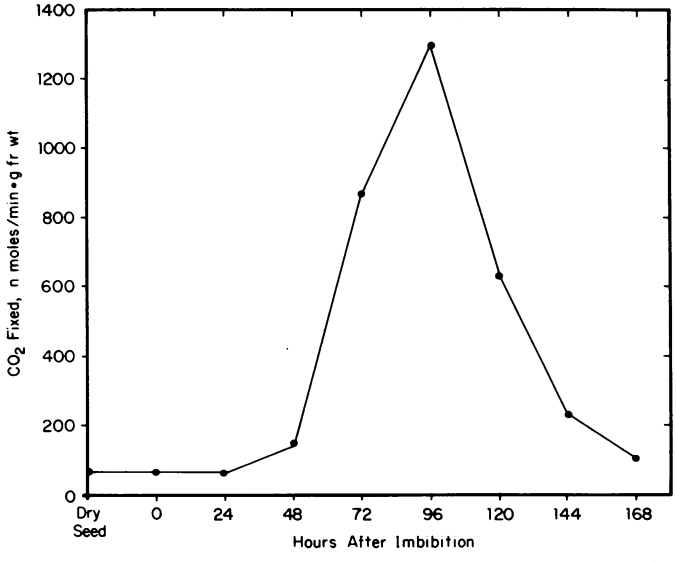

FIG. 2. Development of the RuDP carboxylase activity in endosperms of germinating castor beans in the dark. These seeds were germinated in vermiculite. At 4 days the weight of the endosperm was 0.61 g fresh weight/seed.

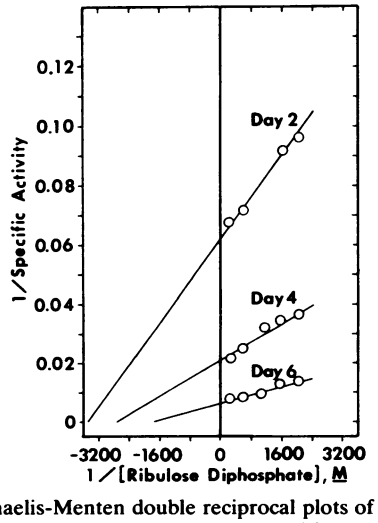

FIG. 3. Michaelis-Menten double reciprocal plots of the RuDP saturation curves in G-200 enzyme fractions isolated from endosperms of 2-, 4-, and 6-day-old germinating castor beans. See "Materials and Methods" for the weight of these endosperms.

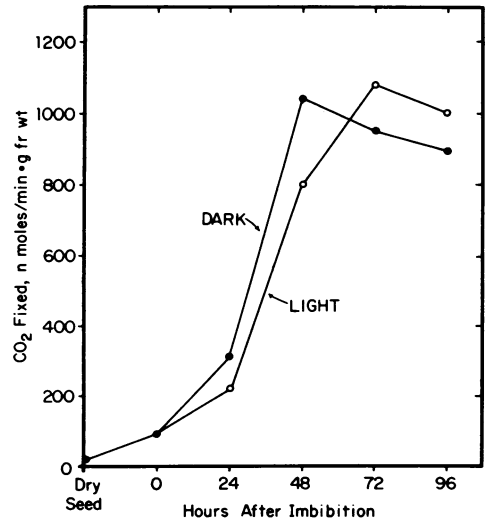

FIG. 4. Effect of light on the development of RuDP carboxylase activity in endosperms of castor beans germinating in Petri dishes. In the seedlings germinating in the dark the weight of the endosperms was 0.629 g fresh weight/bean, after 48 hr. In the seedlings germinating in the light the weight of the endosperm was 0.83 g fresh weight/bean, after 72 hr.

The effect of light on the development of RuDP carboxylase in the castor bean endosperms is shown in Figure 4. In these experiments the imbibed seeds, without seedcoats, were exposed to continuous illumination. During the growth of the castor bean seedlings, light had no promotive effect on the rate of development or on the total level of RuDP carboxylase activity in the endosperms. Apparently phytochrome (Pfr) is not involved in the synthesis of the carboxylase.

The presence of 2 μg/ml CHI or 50 μg/ml CAP completely prevents the development of the carboxylase in the endosperms. These data are consistent with the conclusion that protein synthesis on 80S cytoplasmic ribosomes and on 70S proplastid ribosomes is required for the synthesis of small and large polypeptides of endosperm RuDP carboxylase. These data are also consistent with the data of Margulies (13) and Smillie *et al.* (17) showing the sensitivity of the synthesis of RuDP carboxylase to CHI and CAP, and the data of Kleinkopf *et al.* (10) showing the sensitivity of the synthesis of the large subunit and small subunit of RuDP carboxylase to CAP and CHI, respectively.

RuDP carboxylase has been localized in the endosperm proplastid fraction (15). The equilibrium density of these proplastids from 5-day-old endosperms is 1.22 g cm^3 (14). The fine structure of endosperm proplastids in 5-day-old seedlings is shown in the electron photomicrographs in Figure 5. The proplastids appear as double membrane structures with invaginations of the inner membrane. In these structures there is no crystalline center or prolamellar body formation indicative of the development of proplastids into etioplasts. In electron photomicrographs of dry castor bean seeds, we were able to detect only a few small plastids between extensively developed aleurone grains. The features of the development of endosperm carboxylase is that the synthesis of the large subunit occurs in the proplastid and the synthesis of both types of subunits are synthesized in this nonphotosynthetic tissue in the dark.

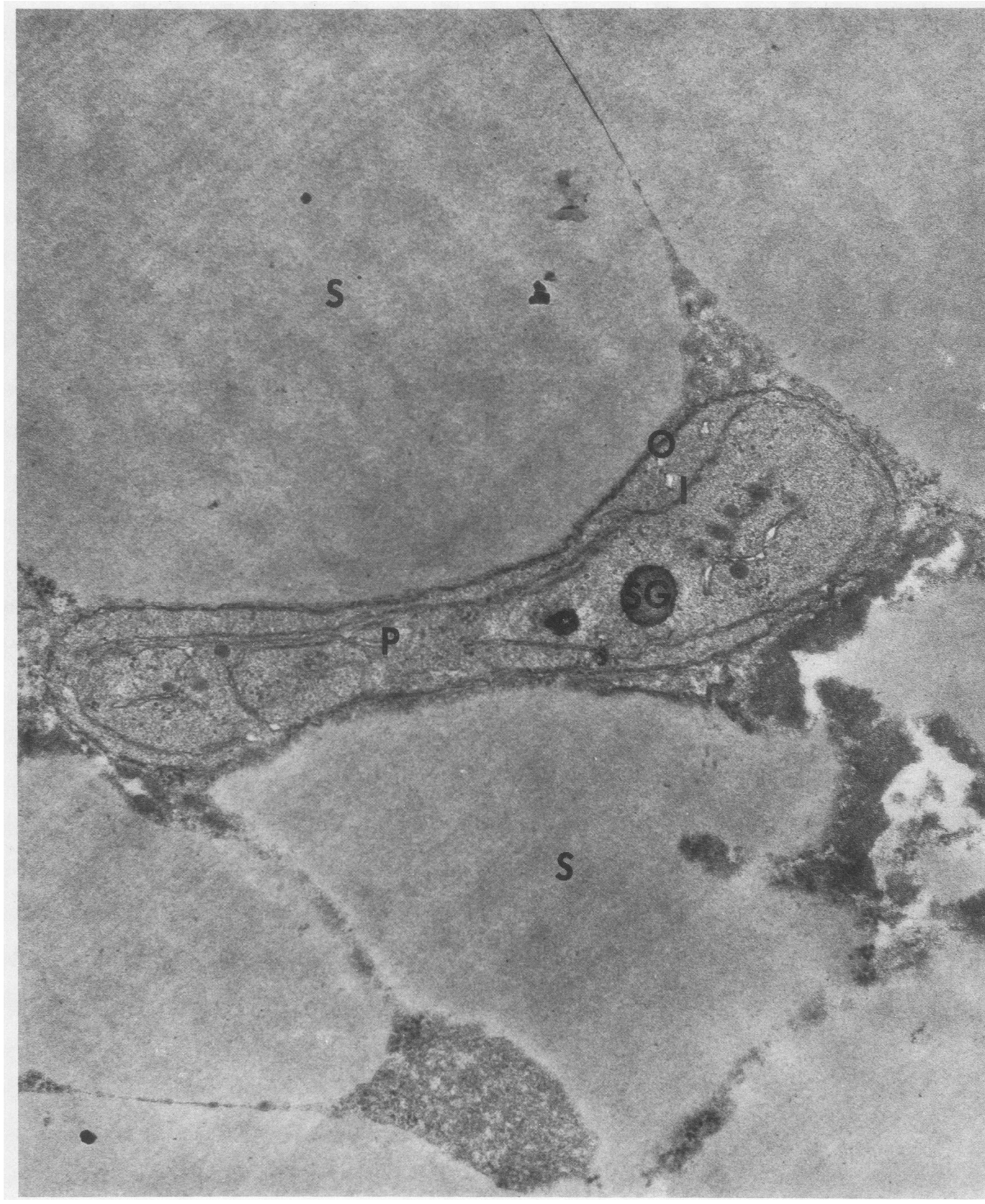

FIG. 5. Electron photomicrographs of proplastids in endosperms of 5-day-old germinating castor beans. S: spherosome; P: proplastid; SG: starch grain; CW: cell wall; O: outer membrane; I: invaginations of inner plastid membrane. ×: 55,800.

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LITERATURE CITED

1. Bahr, J. T. and R. Jensen. 1974. Ribulose diphosphate carboxylase from freshly ruptured spinach chloroplasts having an *in vivo* Km [CO_2]. Plant Physiol. 53: 39–44.
2. Bassham, J. A., P. Sharp, and I. Morris. 1968. The effect of Mg^{2+} concentrations of the pH optimum and Michaelis constants of the spinach chloroplast ribulose diphosphate carboxylase (carboxydismutase). Biochim. Biophys. Acta 153: 898–900.
3. Beevers, H. 1969. Glyoxysomes of castor bean endosperm and their relation to gluconeogenesis. Ann. N. Y. Acad. Sci. 168: 313–324.
4. Benedict, C. R. 1973. The presence of ribulose-1,5-diphosphate carboxylase in the nonphotosynthetic endosperm of germinating castor beans. Plant Physiol. 51: 755–759.
5. Bolley, D. S. and R. L. Holmes. 1958. Inedible oilseed meals. *In:* A. M. Altschul, ed., Processed Plant Protein Foodstuffs. Academic Press, New York, pp. 829–857.
6. Chollet, R. and W. L. Ogren. 1972. Greening in a virescent mutant of maize. II. Enzyme studies. Z. Pflanzenphysiol. Bd. 68. S45–54.
7. Ellis, R. J. 1973. Fraction I protein. Cur. Adv. Plant Sci. 3: 29–38.
8. Haurowitz, F. 1963. The Chemistry and Function of Proteins, 2nd Ed. Academic Press, New York.
9. Kawashima, N. and S. G. Wildman. 1970. Fraction I protein. Annu. Rev. Plant. Physiol. 21: 325–358.
10. Kleinkopf, G. E., R. C. Huffaker, and A. Matheson. 1970. Light-induced *de novo* synthesis of ribulose-1,5-diphosphate carboxylase in greening leaves of barley. Plant Physiol. 46: 416–418.
11. Kuehn, G. D. and B. A. McFadden. 1969. Ribulose-1,5-diphosphate carboxylase from *Hydrogenomonas eutropha* and *Hydrogenomonas facilis*. I. Purification, metallic ion requirements, inhibition and kinetic constants. Biochemistry 8: 2394–2402.
12. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.
13. Margulies, M. M. 1964. Effects of chloramphenicol on light-dependent synthesis of proteins and enzymes of leaves and chloroplasts of *Phaseolus vulgaris*. Plant Physiol. 39: 579–585.
14. Miflin, B. J. and H. Beevers. 1974. Isolation of intact plastids from a range of plant tissues. Plant Physiol. 53: 870–874.
15. Osmond, C. B., T. Akazawa, and H. Beevers. 1975. Localization and properties of ribulose diphosphate carboxylase from castor bean endosperm. Plant Physiol. 55: 226–230.
16. Paulsen, J. M. and M. D. Lane. 1966. Spinach ribulose diphosphate carboxylase. I. Purification and properties of the enzyme. Biochemistry 5: 2350–2357.
17. Smillie, R. M., D. Graham, M. R. Dwyer, A. Grieve, and N. F. Tobin. 1967. Evidence for the synthesis of *in vivo* of proteins of the Calvin cycle and photosynthetic electron transport pathway on chloroplast ribosomes. Biochem. Biophys. Res. Commun. 28: 604.
18. Sugiyama, T., N. Nakayama, and T. Akazawa. 1968. Structure and function of chloroplast proteins. V. Homotropic effect of bicarbonate in RuDP carboxylase reaction and the mechanism of activation by magnesium ions. Arch. Biochem. Biophys. 126: 737–745.
19. Weissbach, A., B. L. Horecker, and J. Hurwitz. 1956. The enzymatic formation of phosphoglyceric acid from ribulose diphosphate and carbon dioxide. J. Biol. Chem. 218: 795–810.
20. Wishnick, M. and M. D. Lane. 1971. Ribulose diphosphate phosphate carboxylase from spinach leaves. Methods Enzymol. 23: 570–577.