Ribulose 1,5-diphosphate carboxylase (RuDP carboxylase) has been shown to be the major component of the Fraction I protein of leaves (5, 6). Protochlorophyllide associated with a protein, i.e. protochlorophyllide holochrome (PCH), has been isolated from etiolated bean leaves (1, 9, 10); upon illumination in vivo or in vitro it is transformed into chlorophyllide holochrome. Several investigators (1, 4, 10) have been unsuccessful in their attempts to separate RuDP carboxylase from PCH and consequently have suggested that the proteins might be identical on the basis of their correspondence with regard to sedimentation velocity, molecular weight and diffusion coefficient.

PCH has so far been extracted only from leaves of etiolated beans although it has been shown to occur in leaves of many types of etiolated plants (e.g. maize). However, it is easy to extract RuDP carboxylase from many of the latter. This suggests that the 2 proteins are distinct but this does not provide unequivocal proof. In the present investigation, RuDP carboxylase and PCH from Phaseolus vulgaris L. variety red kidney were shown to be immunologically distinct.

Materials and Methods

Plant Material. Red kidney beans were planted in vermiculite preequilibrated with water for 12 hr. Plants were either grown in darkness under a green safe light at 28° or were grown in light in a greenhouse.

Preparation of Fraction I Protein. Fraction I protein (FIP) was prepared from freshly collected fully expanded leaves of light-grown plants. The leaves (120 g) were infiltrated with 0.1 M glycine, 0.01 M MgCl₂ adjusted to pH 8.0 with NaOH. They were then homogenized in a Waring Blendor for 1 min with only the addition of 15 mmoles of 2-mercaptoethanol immediately prior to homogenization. The brei was pressed through 2 layers of cheesecloth and the green sap obtained in this manner was centrifuged at 12,000g for 20 min at 4°. The supernatant fluid from this treatment was centrifuged at 4° for 1.5 hr at 226,000g. The supernatant fluid from the high speed centrifugation was decanted and brought to 50% of saturation with either solid ammonium sulfate or a saturated solution of ammonium sulfate. The proteins which precipitated were collected by centrifugation and suspended in a small volume of distilled water. After dialysis overnight against 2.1 of 0.01 M tris-sulfate, pH 8.0, FIP was finally purified by density gradient centrifugation. A 5 ml gradient of 10% to 50% (w/v) sucrose made in 0.01 M tris-sulfate, pH 8.0 was generated in a Spinco SW 40 cellulose nitrate centrifuge tube. One ml of dialyzed protein concentrate was layered on this gradient and the remainder of the tube was filled with tris-sulfate buffer. Centrifugation was carried out for 5 hr at 40,000 rpm in the SW 40 rotor. A highly light-scattering band about one-third of the way down the gradient contained RuDP carboxylase. This was removed with a hypodermic syringe.

Production of Antiserum and Diffusion Analyses. Rabbits which had been pre-bled were injected intravenously with approximately 5 mg of FIP. This initial injection was followed by 5 weekly intramuscular injections, each consisting of a mixture of 5 mg of FIP in 1 ml and in addition 1 ml of Freund's complete adjuvant. Double diffusion plates were made with 2% I onagar in barbitual buffer, pH 8.6 containing 0.2 mg merthiolate per ml and were incubated at room temperature and examined daily (2). Reactions were found to be complete within 48 hr.

Preparation of Crude PCH. Crude PCH was prepared from primary leaves of dark-grown bean plants essentially by the method of Schopfer and Siegelman (8) except that Polyclar AT was omitted from the original homogenizing mixture. The purification was carried out only as far as redissolving the polyethylene glycol precipitate.

Reaction of Anti-FIP With Crude PCH. Aliquots of crude PCH were mixed with normal serum samples taken before inoculation of rabbits with
to transform all the convertible PCH in the preparation to chlorophyllide holochrome.

**Enzyme Assays.** RuDP carboxylase activity was determined by the method of Rabin and Trown (7) as modified by Goldthwaite (personal communication).

**Results**

**Antiserum to FIP.** The result of a double diffusion experiment between antiserum and FIP is shown in Fig. 1. More than 1 precipitin line was found showing that the rabbits had probably produced antibodies to several proteins of FIP. This multiplicity of antibodies did not impair the usefulness of the antiserum, however, as is shown in subsequent experiments.

**Effect of Anti-FIP on PCH Transformation and RuDP Carboxylase Activity.** As has been reported by others, PCH in vitro shows an absorption maximum at 637 nm. After illumination the solution exhibits a maximum at 675 nm attributable to chlorophyllide holochrome newly formed from PCH and some unconvertible protochlorophyllide which absorbs at 630 nm is revealed. The absorption spectra of supernatants to which anti-FIP had been added and to which normal serum had been added are shown in Fig. 2a and 2b, respectively; they are, in all characteristics significant to this investigation, indistinguishable from one another and from the control to which only buffer had been added. Thus, antibody to FIP does not interfere with the photoconversion of PCH in vitro. A precipitate was formed only in the aliquot to which anti-FIP had been added. This event had neither a quantitative nor qualitative effect on the absorption spectrum of the PCH solution. Furthermore, PCH could not be

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**Fig. 1.** A double diffusion experiment demonstrating that rabbits produced antibodies to several proteins of FIP. Wells are charged as indicated, i.e. FIP with purified Fraction I protein; LS with 3 dilutions of leaf sap from light-grown leaves; AFIP with antiserum.

**Fig. 2.** A) The absorption spectrum after centrifugation of crude PCH-FIP solution to which anti-FIP had been added. B) The absorption spectrum after centrifugation of crude PCH-FIP solution to which normal serum had been added. The solid line represents spectra before illumination; the broken line after illumination as described in the text.
detected in the precipitate obtained from the anti-FIP treated preparation, when washed, collected on a Millipore filter and examined with the spectrophotometer. Thus, anti-FIP neither precipitates PCH nor interferes with its photoconversion in solution.

The completeness of removal of FIP from crude PCH-FIP solutions was investigated as described below. The supernatant solutions obtained after centrifugation of a mixture of normal or immune serum with PCH solutions were examined using the double diffusion technique. Fig. 3 shows the results of an experiment in which wells were charged as follows: into the center well was placed anti-FIP (AFIP); wells marked FIP were filled with Fraction I protein obtained from light-grown plants; wells marked CPCH were filled with crude PCH; the well marked BPCH was filled with tricine buffer treated PCH; the well marked NPCH with normal serum treated PCH; and the well marked APCH with immune serum treated PCH. Several precipitin lines formed in all intersecting areas except those between AFIP and APCH. The single faint precipitin line present between these 2 wells is attributed to an antigen-antibody reaction of low avidity (3). The absence of major precipitin bands in the APCH-AFIP reaction area confirmed the expectation that treatment with antiserum caused fairly complete precipitation of RuDP carboxylase, the major component of FIP. As a further check on the completeness of precipitation of RuDP carboxylase, the supernatant solutions were tested for RuDP carboxylase activity. Seven cpm were incorporated into phosphoglyceric acid using APCH as a source of enzyme; 89 cpm using NPCH; and 75 cpm using BPCH. Thus, PCH solutions which had been treated with antiserum had no or virtually no RuDP carboxylase activity, in agreement with the observations of the gel diffusion experiments.

**Conclusion**

Serum containing antibodies to FIP precipitates FIP but neither precipitates PCH nor interferes qualitatively or quantitatively with the photoconversion of PCH to chlorophyllide holochrome in vitro. Thus, the protein of PCH and that of FIP, whose principal constituent is RuDP carboxylase, are different. An immunochemical step might permit the preparation of purer protochlorophyllide holochrome than has been available to date.

**Literature Cited**