

# Localization and Properties of Ribulose Diphosphate Carboxylase from Castor Bean Endosperm<sup>1</sup>

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## ABSTRACT

A substantial portion of the ribulose 1,5-diphosphate carboxylase activity in the endosperm of germinating castor beans (*Ricinus communis* var. Hale) is recovered in the proplastid fraction. The partially purified enzyme shows homology with the enzyme from spinach (*Spinacia oleracea*) leaves, as evidenced by its reaction against antibodies to the native spinach enzyme and to its catalytic subunit. The enzyme from the endosperm of castor beans has a molecular weight of about 500,000 and, with the exception of a higher affinity for ribulose 1,5-diphosphate, has similar kinetic properties to the spinach enzyme. The castor bean carboxylase is inhibited by oxygen and also displays ribulose 1,5-diphosphate oxygenase activity with an optimum at pH 7.5.

## MATERIALS AND METHODS

Seeds of castor bean (*Ricinus communis* var. Hale) were soaked in flowing tap water for 24 hr and then sown in flats of H<sub>2</sub>O-saturated vermiculite. The seeds were germinated in the dark in a humidified cabinet at 30 C. The testae and cotyledons were removed and the washed endosperm halves were extracted as required.

Changes in total enzyme activity during germination were determined in the 40,000g supernatant of extracts prepared from 10 castor beans homogenized in 20 ml of 100 mM Tricine, pH 7.5, containing 10 mM MgCl<sub>2</sub> and 2 mM DTT, with a glass homogenizer. RuDP carboxylase was partially purified from larger scale preparations in which about 70 g of castor beans were extracted in 150 mM tris buffer, pH 8.0, containing 15 mM MgCl<sub>2</sub> and 3 mM DTT. The 40,000g supernatant was fractionated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and the 25 to 55% saturation fraction (containing about 90% of the total activity) was resuspended in a small volume of the extracting buffer. After dialysis for 12 hr against 50 mM tris buffer, pH 7.8, containing 5 mM MgCl<sub>2</sub> and 1 mM DTT, the soluble protein was layered on a 7 to 27% linear sucrose gradient (33 ml) containing the same buffer and centrifuged for 24 hr at 23,500 rpm (SW 27 rotor, RC-2B). RuDP carboxylase was assayed in 50- $\mu$ l samples from the 1.2-ml fractions collected from the gradient and was recovered from the peak fractions by precipitation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> at 70% saturation.

In other experiments, the 25 to 55% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitate was redissolved in 25 mM tris, pH 7.5, containing 100 mM KCl and 0.1 mM EDTA, and was applied to a 2.5  $\times$  75 cm column of Sephadex G-200 equilibrated with the same buffer. RuDP carboxylase activity was measured in fractions eluted from this column and the active fraction concentrated in an Amicon filter. The crude (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fraction and the Sephadex purified enzyme were examined by polyacrylamide gel electrophoresis at pH 8.9 (9). Some gels were stained to localize the proteins; others were embedded in agar plates and were challenged with antibodies prepared against purified native spinach RuDP carboxylase (Anti-N) and against purified subunit A of spinach RuDP carboxylase (Anti-A) as described by Nishimura and Akazawa (16). Mobility of the purified carboxylase in polyacrylamide gels was compared to that of spinach RuDP carboxylase and used to estimate mol wt (11).

Organelle preparations were obtained by chopping with razor blades endosperm tissue in 20% sucrose containing 100 mM Tricine (pH 7.5), 10 mM KCl, 1 mM EDTA, and 1 mM DTT. The whole homogenate, after filtration through 2 layers of cheesecloth, or the 270g pellet, resuspended in extracting medium, was applied to a 20 to 60% linear sucrose gradient containing the same buffer and additives as the extracting buffer. Ten ml of homogenate were applied to a 35-ml gradient

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Ribulose diphosphate carboxylase activity was detected in the endosperm of germinating castor beans by Benedict (4). He proposed that the enzyme might be localized in the proplastids of the endosperm tissue and the experiments described here support this proposal. (It should be emphasized that although the organelles in castor bean endosperm referred to here as proplastids meet the implied morphological and enzymic criteria, they do not develop into chloroplasts; the white endosperm tissue is completely consumed during germination and never becomes photosynthetic.) In recent years RuDP<sup>4</sup> carboxylase has been shown to have a second enzyme function. The enzyme will fix molecular O<sub>2</sub> in place of CO<sub>2</sub>, and this enzymatic function, identified in a range of photosynthetic organisms, has been described as RuDP oxygenase (1, 2, 18). The present experiments show RuDP carboxylase of castor bean endosperm to be essentially similar to that of higher plant leaves and that the enzyme from non-photosynthetic tissue also has RuDP oxygenase activity.

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<sup>4</sup> Abbreviations: RuDP: ribulose 1,5-diphosphate; DTT: dithiothreitol.

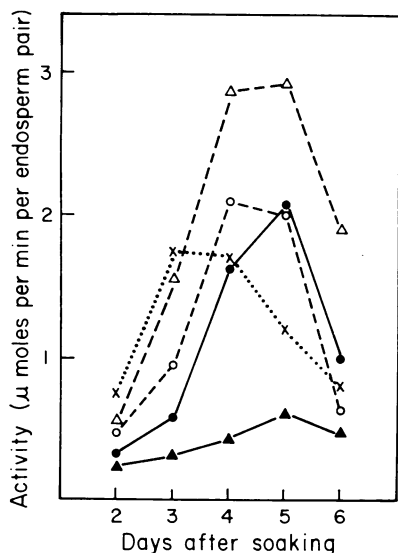


FIG. 1. Changes in enzyme activity during germination of castor beans. Ribulose diphosphate carboxylase  $\times 10$  (●); triosephosphate isomerase  $\times 10^{-2}$  (▲); fructose 1,6-diphosphatase (×); isocitrate lyase (○) and fumarase (Δ).

over a 6-ml cushion of 60% sucrose. Gradients were centrifuged for 4 hr at 21,000 rpm (SW 21, RC-2B) and then cut into 1.2-ml fractions. In some experiments, a modified gradient of the type described by Mifflin and Beevers (15) was employed. The gradient and the 20% sucrose extracting medium contained Ficoll, Dextran, and albumin as described earlier (15). These gradients were centrifuged for 5 min at 4,000 rpm, 15 min at 10,000 rpm, and allowed to decelerate to 5,000 rpm before application of the brake.

RuDP carboxylase was assayed by the incorporation of  $^{14}\text{CO}_2$  into acid-stable products (4) and by the coupled spectrophotometric assay (2). Both methods yielded similar results but all the data presented were obtained with the  $^{14}\text{CO}_2$  assay. Samples (50  $\mu\text{l}$ ) of gradient fractions were used to initiate the reaction in 0.1 to 0.5 ml of a reaction mixture containing 150 mM tris (pH 8.0), 15 mM  $\text{MgCl}_2$ , 50 mM  $\text{NaH}^{14}\text{CO}_3$  (0.2  $\mu\text{Ci}/\mu\text{mole}$ ), and 1 mM RuDP. After 30 min at 36 C the reaction was stopped with 0.5 ml of glacial acetic acid, the mixture was boiled for 10 min on a hot plate, and after cooling, was counted in 5 ml of a suitable scintillant. The enzyme was not preincubated with  $\text{HCO}_3^-$  or  $\text{Mg}^{2+}$ , and the reaction was linear for 30 min. Kinetic studies were carried out at 25 C using a similar assay system.

RuDP oxygenase was assayed using a Rank polarographic  $\text{O}_2$  electrode (1). Buffers were prepared from tris-HCl which was purged for 2 hr with  $\text{CO}_2$ -free  $\text{N}_2$  at pH 2.8 before adjusting pH with NaOH prepared from freshly washed pellets. Prior to assay the buffer mixtures (200 mM tris, 20 mM  $\text{MgCl}_2$ ) were equilibrated with  $\text{CO}_2$ -free air at 30 C. The assay, which contained 115 mM tris, 11.5 mM  $\text{MgCl}_2$ , and between 0.5 and 10 mg of enzyme protein, was initiated by the addition of (0.4 mM) RuDP.

Triose-P isomerase was assayed in 100 mM tris, pH 7.4, containing 2.2 mM DTT, 0.12 mM NADH, 1.25 units of  $\alpha$ -glycerophosphate dehydrogenase, and was initiated by addition of 7.7 mM glyceraldehyde-3-P. Fructose 1,6-diphosphatase was assayed in 100 mM tris, pH 7.0, containing 10 mM  $\text{MgCl}_2$ , 0.12 mM NADP, 2.5 units each of glucose-P isomerase and glucose-6-P dehydrogenase and was initiated by the addition of 0.8 mM fructose-1,6-DiP. Isocitrate-lyase was assayed in 100

mM tris, pH 7, containing 10 mM  $\text{MgCl}_2$ , 12 mM DL-isocitrate, 12 mM phenylhydrazine-HCl and was initiated with enzyme. Fumarase was assayed in 100 mM phosphate buffer, pH 7.3, containing 22 mM L-malate and was initiated with enzyme.

## RESULTS AND DISCUSSION

The development of RuDP carboxylase activity in the endosperm of germinating castor bean, shown in Figure 1, is substantially similar to that described by Benedict (4). Figure 1 shows that the maximum activity of RuDP carboxylase corresponds to the peak activity of fumarase (mitochondrial marker), isocitrate-lyase (glyoxysomal marker), and of triose-P isomerase, an enzyme partly associated with the proplastids in this tissue (15). Fructose 1,6-diphosphatase, an enzyme which to date is not known to be associated with organelles in this tissue (13), exhibited a distinctly different pattern of activity during germination.

**Localization of Endosperm RuDP Carboxylase.** A variable proportion of the RuDP carboxylase was associated with the proplastid fraction on linear gradients prepared from 3.5- to 5-day-old castor bean endosperm. Figure 2 shows that about 45% of the RuDP carboxylase in the whole homogenate of 5-

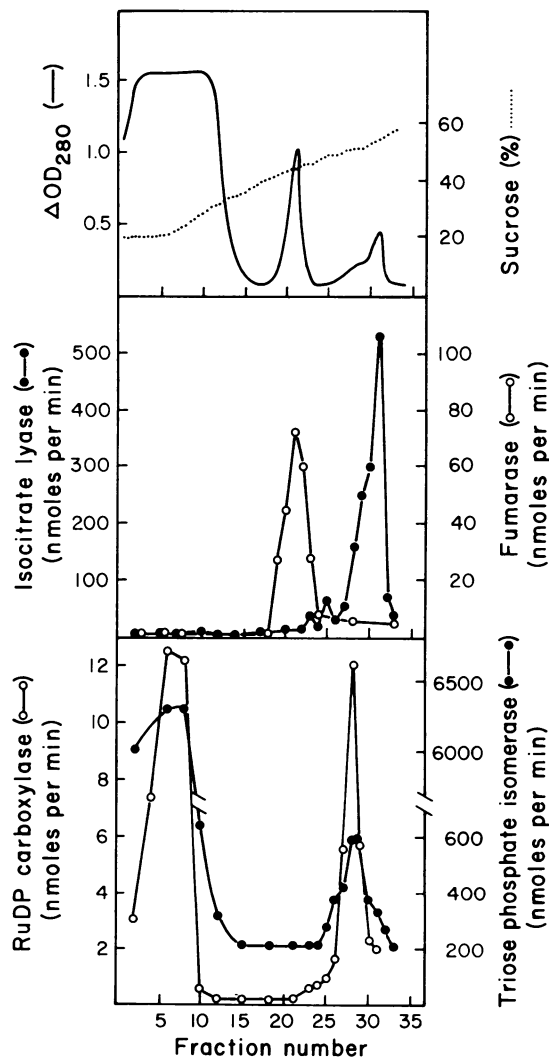


FIG. 2. Equilibrium density gradient separation of organelles from whole homogenate of 5-day endosperm of germinating castor beans.

day endosperm was associated with an organelle fraction of density 1.23 g/cc which was clearly separated from the mitochondria and glyoxysomes. In other experiments, as little as 5% of the activity in 3-day endosperm and as much as 70% of the activity of 5-day endosperm prepared in 12% sucrose, was retained in this region of the gradient. If the crude homogenate was first centrifuged for 10 min at 270g, a substantial portion of the total RuDP carboxylase activity was sedimented. When the 270g pellet was resuspended in the extracting medium and applied to the linear gradient, about 80% of the total activity moved to equilibrium density 1.23 g/cc, identically to that shown in Figure 2. Triose-P isomerase activity in gradients from the 270g pellet coincided with that of RuDP carboxylase, but in gradients prepared from whole homogenates, less than 5% of the triose-P isomerase activity was found in this peak. Fructose 1,6-diphosphatase activity showed no association with the proplastids or any other organelle.

The complex gradient devised by Mifflin and Beevers (15) permits separation of organelles by a combination of sedimentation velocity and density equilibration. Although most suited to studies with photosynthetic tissues, it permits independent resolution of organelles in castor bean endosperm. When whole homogenates or the resuspended 270g pellet of 4-day endosperm preparations were centrifuged on these gradients, a distinct peak of RuDP carboxylase activity moved rapidly into the denser region of the gradient, well in advance of the glyoxysomes (Fig. 3). Although the bulk of the carboxylase remained in the soluble phase, these experiments provide convincing evidence that at least part of this enzyme is associated with the fraction commonly identified as the proplastids. The behavior of RuDP carboxylase on these complex gradients is similar to that reported previously for triose-P

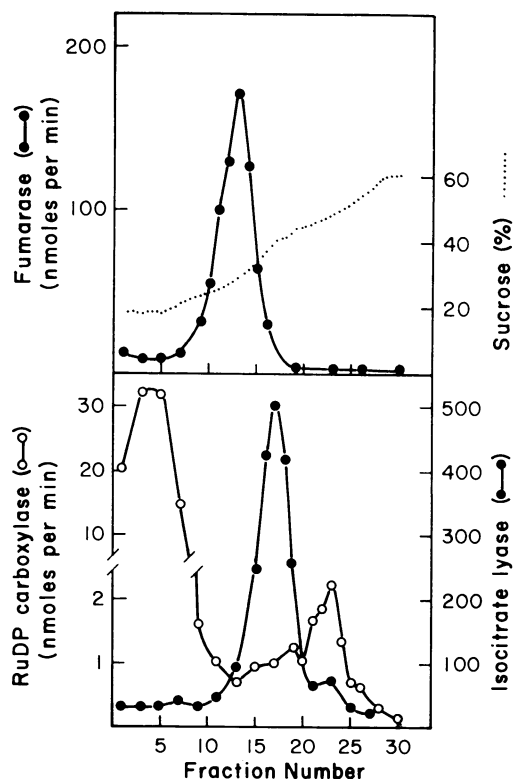


FIG. 3. Combined sedimentation velocity and equilibrium density gradient separation of organelles from whole homogenate of 4-day endosperm of germinating castor beans.

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

Enzyme Fraction	Specific Activity	
	Expt 1	Expt 2
Crude extract	3.2	10.8
Sucrose density centrifugation	24.9	37.5
Elution from Sephadex G-200	17.0	

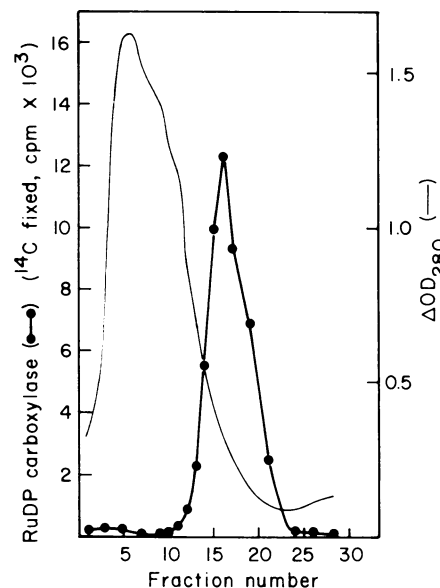


FIG. 4. Partial purification of castor bean endosperm ribulose diphosphate carboxylase by density gradient centrifugation of the resuspended 25 to 55%  $(\text{NH}_4)_2\text{SO}_4$  precipitate.

isomerase. It is evident in these studies that the proplastids are much less robust than either the mitochondria or glyoxysomes, and that isolation of intact proplastids in high yield may require conditions less than optimal for the other organelles.

**Partial Purification of Endosperm RuDP Carboxylase.** The specific activity of the carboxylase in the crude castor bean extracts used here is about one-tenth that reported by Benedict (4). Simple purification procedures were used in order to concentrate the carboxylase for RuDP oxygenase assays and to remove major contaminating enzymes prior to kinetic analysis and serological examination. Ammonium sulfate precipitation, followed by density gradient centrifugation or elution from Sephadex G-200, resulted in a 5-fold increase in specific activity (Table I).

Figure 4 shows that the RuDP carboxylase activity moved away from most of the soluble protein applied to a linear sucrose gradient. This centrifugation pattern is similar to that observed in crude extracts of photosynthetic tissues (2, 10) and suggests that the endosperm RuDP carboxylase is a protein of relatively high mol wt.

The protein recovered from the fractions of peak activity contained one slow moving band and only traces of more mobile proteins when subjected to polyacrylamide gel electrophoresis. RuDP carboxylase from the sucrose gradients was used for the kinetic studies described below.

Figure 5 compares the patterns of proteins found on 7% polyacrylamide gels following electrophoresis of the crude  $(\text{NH}_4)_2\text{SO}_4$  fraction (25–55% saturation) and the eluate from

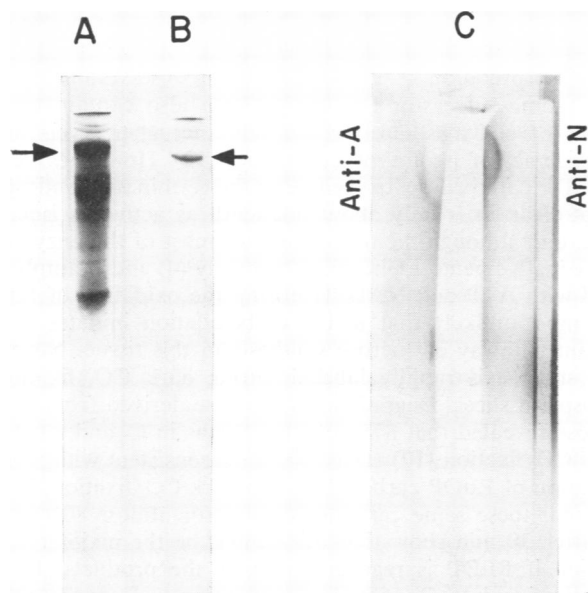


FIG. 5. Polyacrylamide gel electrophoresis of crude (A) and purified (B) preparations of castor bean endosperm RuDP carboxylase and double immunodiffusion (C) of purified enzyme challenged with rabbit antisera prepared against each of spinach RuDP carboxylase (Anti-N) and its catalytic larger subunit (Anti-A). About 60  $\mu\text{g}$  of the endosperm RuDP carboxylase preparations were applied to polyacrylamide gel, 7.5%, pH 8.5. After the electrophoretic run, the proteins in the gel were stained with Coomassie brilliant blue (enzyme bands are indicated by arrows) (A and B). The electrophoresed gel of the purified enzyme was buried into a plate of 1% agar in 0.5 M tris-HCl buffer (pH 7). Afterwards, 0.2 mg of  $\gamma$ -globulin fraction of Anti-N was placed in the right well and 0.3 mg of Anti-A was placed in the left well. Immunoprecipitation lines formed during 72 hr incubation at 20 C were stained with Amidoblack 10B (C).

the Sephadex G-200 column. A single, slow moving band was observed in the preparation recovered from the Sephadex. The mobility of this protein band in gels of 4, 5, 6, and 7.5% was measured and the slope of the plot of  $100 \log (R_m \times 100)$  against gel concentration was determined (Fig. 6). The slope found for castor bean endosperm RuDP carboxylase was 23.5 and compares with a slope for purified spinach RuDP carboxylase of 23.7. It is clear that the enzyme from castor bean endosperm has a mol wt in the region of 500,000 and is substantially similar to the spinach leaf enzyme.

Figure 5 also shows that when the unstained gels prepared with purified castor bean endosperm RuDP carboxylase were challenged with antibodies to the spinach leaf enzyme, prominent precipitin bands developed in the region of the gel corresponding to the single protein band. The castor bean endosperm enzyme reacted against antibodies to the native enzyme (Anti-N) and to the large catalytic subunit A (Anti-A) (16). This reaction shows the substantial homology between the native enzyme and catalytic subunits of the photosynthetic enzyme and the endosperm enzyme. Spinach leaf enzyme and castor bean endosperm enzyme were also challenged with Anti-N serum in a conventional Ouchterlony procedure and again the precipitin bands indicated substantial identity of the RuDP carboxylase from the two sources.

**Some Properties of Endosperm RuDP Carboxylase.** Some kinetic properties of partially purified RuDP carboxylase from the endosperm of castor bean are shown in Table II where they are compared with those of spinach leaf enzyme. The enzyme from endosperm has a significantly lower apparent

$K_m$  for RuDP but is otherwise similar to that of the purified enzyme from spinach when assayed in the same way. No evidence was obtained for the activation or inhibition of the enzyme by fructose-6-P or fructose diP in assays containing 0.5 or 1 mM  $\text{NaHCO}_3$  and  $\text{MgCl}_2$ . Whether this further distinction from the spinach leaf enzyme (8) is related to the low  $K_m$  for RuDP remains to be determined.

RuDP carboxylase from the castor bean endosperm is inhibited by  $\text{O}_2$ , particularly at low  $\text{CO}_2$  concentrations (Table III). The inhibition due to  $\text{O}_2$  appears to be competitive in character, as it is in higher plant preparations (7).

Partially purified RuDP carboxylase from the castor bean

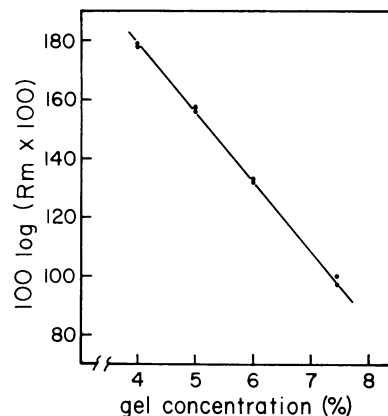


FIG. 6. Mobility of purified endosperm RuDP carboxylase on polyacrylamide gels of increasing concentration. The purified endosperm enzyme protein was subjected to electrophoresis at different polyacrylamide gel concentrations (4, 5, 6, and 7.5%) according to the method of Hedrick and Smith (11), and the slope obtained was used to estimate the mol wt of the enzyme protein using a calibration curve, mol wt versus slope, using the following marker proteins: chymotrypsinogen ( $2.6 \times 10^4$ ), albumin (Rinderserum, monomer) ( $6.7 \times 10^4$ ), dimer ( $13.4 \times 10^4$ ), trimer ( $20.1 \times 10^4$ ), hexokinase ( $9.9 \times 10^4$ ), and aldolase ( $1.6 \times 10^5$ ).

Table II. Apparent  $K_m$  for Substrates and  $\text{Mg}^{2+}$

The values are shown by castor bean endosperm ribulose diphosphate carboxylase partially purified by  $(\text{NH}_4)_2\text{SO}_4$  precipitation and sucrose density centrifugation.

Substrate	Castor Bean Endosperm	Spinach Leaf <sup>1</sup>
Ribulose diphosphate	$1.7 \times 10^{-5}$	$1-2.5 \times 10^{-4}$
$\text{HCO}_3^-$	$1.6 \times 10^{-2}$	$1-2 \times 10^{-2}$
$\text{Mg}^{2+}$	$5 \times 10^{-4}$	$1 \times 10^{-3}$

<sup>1</sup> Buchanan and Schürman (8).

Table III. Inhibition of Crude Castor Bean Endosperm Ribulose Diphosphate Carboxylase by Oxygen

The dialyzed  $(\text{NH}_4)_2\text{SO}_4$  precipitate was the source of enzyme.

Equilibrium Gas Phase	$\text{HCO}_3^-$ Concn (mM)		
	0.25	1.0	5.0
	<i>nmoles <math>^{14}\text{CO}_2</math> fixed</i>		
100% $\text{N}_2$	7.9	32.6	123.1
Air	7.2	25.6	116.5
100% $\text{O}_2$	3.1	18.7	78.5

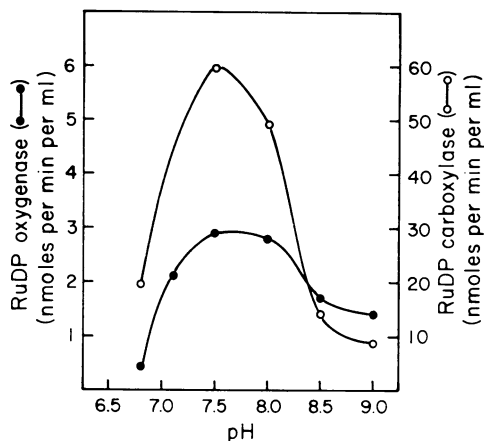


FIG. 7. Ribulose diphosphate carboxylase and oxygenase activity in the 25 to 55%  $(\text{NH}_4)_2\text{SO}_4$  fraction from endosperm of germinating castor beans. Different preparations were used for the carboxylase and oxygenase assays shown.

endosperm shows RuDP oxygenase activity. The oxygenase activity at optimal pH and in 21%  $\text{O}_2$  was only 0.9% of the carboxylase activity, a ratio similar to that found in the marine alga *Halimeda cylindracea* (1). In comparison, the activity of oxygenase extracts of *Atriplex glabriuscula* was 2.9% of carboxylase activity when assayed at optimal pH and 21%  $\text{O}_2$  in a parallel experiment. RuDP oxygenase activity in extracts of castor bean endosperm was unusual in that the pH optimum of the oxygenase was the same as that of carboxylase (Fig. 7). In higher plants and in marine alga, the pH optima of RuDP carboxylase and RuDP oxygenase prepared by ammonium sulfate fractionation and density gradient centrifugation are at least one pH unit apart. RuDP oxygenase in *A. glabriuscula* extracts assayed under comparable conditions showed an optimum at pH 9.0, similar to that observed in spinach extracts, in *Halimeda cylindracea* and in *Chromatium* (1, 2, 18). At pH 7.5 the  $\text{O}_2$  uptake initiated by the addition of 0.5  $\mu\text{mole}$  of RuDP to castor bean preparations was completely abolished by the addition of 10  $\mu\text{moles}$  of  $\text{NaHCO}_3$ . This eliminates the possibility that the  $\text{O}_2$  uptake is due to contaminating glycolate and glycolate oxidase.

### CONCLUSIONS

Although the specific activity of the RuDP carboxylase in crude extracts of the nonphotosynthetic castor bean endosperm (17–37 nmoles/min·mg protein) is only ~2% of that in green leaf extracts the properties of the enzyme are similar to that from photosynthetic tissues. Of particular interest is the observation that the endosperm carboxylase also shows RuDP oxygenase activity. The endosperm carboxylase does not function in a photosynthetic environment (*i.e.*, it does not function in association with an  $\text{O}_2$ -evolving system) and the observation that it also shows RuDP oxygenase activity is consistent with the notion that this activity may be a consequence of the RuDP carboxylation mechanism itself (7, 14). The correspondence of pH optima for the two enzyme activities in castor bean endosperm is of some interest and it should be noted that the pH optimum of oxygenase activity in lysed chloroplasts is substantially lower than that of purified fraction 1 protein (Badger and Andrews, unpublished observations).

The localization of a substantial part of the endosperm RuDP carboxylase in the proplastids and the low specific ac-

tivity suggest incomplete repression of this enzyme system, normally restricted to the chloroplasts of photosynthetic tissues. This interpretation is in line with the observation that the glyoxysomes of castor bean endosperm also contain low activities of enzymes normally present in higher levels in the peroxisomes of photosynthetic tissues (12). However, the role of RuDP carboxylase/oxygenase in the germinating endosperm is not clear. It is only about one-tenth as active as isocitrate lyase, and although the tissue contains most of the enzymes of the reductive pentose phosphate pathway (4) and is amply supplied with ATP and NADPH during the oxidation of fat (3), it is most unlikely that RuDP carboxylation initiates an important pathway of sucrose synthesis in this tissue. Nevertheless, sucrose is rapidly labeled during dark  $\text{CO}_2$  fixation in endosperm slices. Degradation of hexose derived from labeled sucrose revealed that 87% of the  $^{14}\text{C}$  was in  $\text{C}_3$  and  $\text{C}_4$  after 5 min  $^{14}\text{CO}_2$  fixation (17). These data are consistent with the participation of RuDP carboxylation in dark  $\text{CO}_2$  fixation, but the fact that these same carbons account for almost 80% of the  $^{14}\text{C}$  after 30 min show that this cannot be the major route of fixation if RuDP is regenerated from the products. Instead,  $\text{CO}_2$  fixation into  $\text{C}_4$  of malate, spreading to  $\text{C}_1$ , and reversed glycolysis from PEP seems to be the major pathway, as indicated earlier (5).

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