Ribulose bisphosphate carboxylase/oxygenase was isolated and crystallized from eight plant species. Crystals grew from either of two similar sets of crystallizing conditions: crystals of the enzyme from alfalfa, corn, cotton, potato, spinach, tobacco, and tomato grew from a mixture of ammonium sulfate and polyethylene glycol 6000 as a precipitant, and those from potato, tobacco (both *Nicotiana sylvestris* and *Nicotiana tabacum*), and tomato grew from a mixture of ammonium sulfate and phosphate. Crystals of the enzyme from potato and tomato were large enough to characterize by x-ray diffraction and were found to have the Form III structure, previously reported for crystals of ribulose bisphosphate carboxylase/oxygenase from *N. tabacum*. For crystalline material from several species, both carboxylase and oxygenase activities have been assayed and copper and iron contents have been determined. The possible significance of the observed general conditions of crystallization of this enzyme is discussed.

Carboxylase° initiates the Calvin cycle of photosynthesis by condensing CO₂ with ribulose bisphosphate (1). It has been termed the most abundant protein in the world (2) and is the principal protein of chloroplasts, where it may exist in the crystalline state (3, 11, 12). Crystals of carboxylase have previously been grown from tobacco (4-7), spinach (8), and from at least two microorganisms (9, 10). X-ray crystal structures from tobacco have been of three different polymorphs, corresponding to various conditions of the crystallization. Forms I and II yielded information on the molecular symmetry and shape of carboxylase (5, 6) and Form III is suitable for the structural studies at medium to high resolution which are currently in progress (3). Spinach crystals reported earlier were not large enough for characterization by x-ray diffraction. The crystals reported here are those of carboxylase from spinach and tobacco grown under new conditions, as well as those of the enzyme from five other species, not previously crystallized.

Crystallization of an enzyme normally has dual significance. It suggests some degree of purity and, with favorable specimens, it opens the way to structural studies of the enzyme by diffraction methods. For carboxylase, crystallization has three additional implications: 1) in vitro crystals may be related to the crystallites found in chloroplasts (11, 12), 2) the fact that a given crystallizing condition produces crystals from a variety of species suggests that some external amino acid residues of the carboxylase molecule are highly conserved, and 3) the availability of crystalline enzyme from a variety of species permits assay of both the carboxylase and oxygenase functions, allowing one to investigate the relative levels of these activities in different species and to determine whether the two activities are invariably linked. The availability of crystals also permits systematic measurements of metal contents of the various carboxylases.

**EXPERIMENTAL PROCEDURES**

**Purification of Enzyme**

Carboxylase was prepared from freshly harvested, washed, and chilled leaves of 10-day-old corn, 4- to 6-week-old potato, 4- to 6-week-old tomato, 8- to 10-week-old alfalfa, 8- to 10-week-old tobacco, and 6- to 10-week-old cotton. The enzyme from all of the species listed here, with the exception of cotton, was purified as previously reported for spinach (8).

Carboxylase from cotton was prepared as follows. Cotton leaves (1 g fresh weight per 8.0 ml medium) were homogenized with a Waring Blender in 0.05 M borate buffer, pH 7.8, 0.2 M NaCl, 0.02 M Na₂SO₄. Solid ammonium sulfate was added to 30% saturation at 4°C. The homogenate was filtered through a layer of Miracloth and two layers of cheesecloth. The filtrate was centrifuged (14,600 × g, 30 min) and the supernatant was passed through 0.05 M Tris-HCl (pH 7.8) containing 0.2 M NaCl and 5 × 10⁻³ M EDTA. The protein peak was collected and dialyzed overnight against the Tris buffer (0.05 M Tris-HCl, pH 7.8 containing 0.2 M NaCl and 5 × 10⁻³ M EDTA) and was applied to a 5 to 35% sucrose gradient containing the same buffer. Centrifugation was carried out at 4°C in a zonal rotor (18 h, 40,000 rpm, Beckman Ti-14 rotor). The symmetrical 18 S protein peak was collected and dialyzed against the Tris buffer for 6 h at 12°C, and the protein was precipitated with 50% ammonium sulfate.

**Crystallization Methods**

**Method 1: Near Equilibrium Vapor Diffusion with PEG 6000**

The 50% ammonium sulfate precipitates from the various species were dissolved in Tris buffer (0.05 M Tris-HCl, pH 7.8, containing 0.2 M NaCl and 5 × 10⁻³ M EDTA) and dialyzed overnight against Buffer A (0.05 M potassium phosphate, pH 7.2). The protein was diluted with Buffer A to 10 mg/ml (assuming A₂₈₀ = 7.0 for all species except spinach, where A₂₈₀ = 6.1) and centrifuged. A 25- to 50-μl droplet was then mixed into a spot plate depression with an equal volume of Buffer
Crystallization of Rubisco

B (0.05 mM KH₂PO₄, adjusted to pH 5.4 with KOH) containing 8% (w/v) PEG 6000 (Fisher Scientific Co., Fairlawn, NJ). The pH of this mixture was 6.7. This mixture is called the "mixed solution" below. The spot plates, supported in a sealed container above a 150-ml reservoir of 8% PEG 6000 in Buffer B, were left undisturbed for 2 to 3 weeks. In some experiments, the pH of the PEG 6000 solution was varied (by adjusting with KOH) from 4.8 to 7.0, resulting in a final pH, after mixing with protein solution, from 6.6 to 7.0. In these experiments, the reservoir solution was 8% PEG 6000 in Buffer B, pH 5.4.

Method 1a: Variation in Method 1 for Potato and Tobacco Proteins—The method is identical to Method 1, except that Buffer C (0.05 M potassium phosphate, pH 7.2, with 0.01 M NaCl) replaces Buffer A.

Method 2: Near Equilibrium Vapor Diffusion with Potassium Phosphate/Ammonium Sulfate Solutions—Carboxylase from tobacco and potato were diluted with Buffer C to 10 mg/ml and centrifuged; tomato carboxylase was diluted with Buffer A and centrifuged. This protein solution was mixed thoroughly in a spot plate depression with an equal volume of 0.375 M ammonium sulfate in Buffer B. The pH of the mixed solution was 6.7. The spot plate containing the solution was placed in a sealed container above a reservoir of 0.375 M ammonium sulfate in Buffer B. In some experiments, the pH of the ammonium sulfate solution was varied (by adjusting with KOH) from 4.8 to 7.0, resulting in a final pH, after mixing with protein solution, from 6.6 to 7.0. In these experiments, the reservoir solution was 0.375 M ammonium sulfate in Buffer B. For some species, Methods 1 and 2 were carried out at 4°C and for others they were carried out at room temperature.

Method 3: Preparative Crystallization by Vapor Diffusion with PEG 6000 as Precipitant—Three milliliters of each of 8% PEG in Buffer B (or in water) and carboxylase (10 mg/ml in Buffer A or C) were mixed in a Petri dish (35 × 10 mm). The dish was placed in a sealed, evacuated desiccator, containing silica gel for 12 to 24 h at room temperature.

Method 4: Preparative Crystallization of Potato Protein by Dialysis against Potassium Phosphate/Ammonium Sulfate Mixtures—Carboxylase at 10 mg/ml in Buffer C was dialyzed at room temperature for 48 h against Buffer B containing 0.375 M ammonium sulfate, adjusted to pH 5.4.

Carboxylase Assay—RuBP was purchased from Sigma (St. Louis, MO). Assays were recommended by Jensen and Bahr (13). The assay mixtures contained the following components: 25 mM (2-hydroxyethyl)piperazine-ethanesulfonic acid, pH 8.0; 20 mM MgCl₂; 2.5 mM dithiothreitol; 20 mM [¹⁴C]NaHCO₃ (0.3 to 0.6 Ci/mmol); 0.5 mM RuBP; and 38 to 75 µg of the enzyme. The total reaction volume was 500 µl, and the assays were run at 25°C. The heat-activated (50°C, 20 min) enzyme was preincubated in the assay mixture (25°C, 4 min), RuBP was added, and the reaction was terminated after 30 s by addition of 100 µl of 2 N HCl. Enzyme [¹⁴CO₂] was released by evaporation of the sample to dryness. After two cycles of activation of 200 µl of 2 N HCl and evaporation to dryness, [¹⁴CO₂] fixation was measured as acid-stable radioactivity by scintillation counting.

For an assay using this protocol and containing 50 µg of enzyme and using [¹⁴C]NaHCO₃, with a specific activity of 0.50 Ci/mmol, 25,250 cpm (above background) incorporated during a 30-min incubation was equivalent to 1 µmol of CO₂ fixed per mg of enzyme per min. Radioactivity was measured using a Beckman liquid scintillation counter with an efficiency of 95% for [¹⁴C].

RuBP Oxygenase Assay—Oxygenase assays were carried out by modifications of recommended procedures (14). The activation medium consists of 10 mM Tris-HCl, pH 8.6; 20 mM MgCl₂; 10 mM NaHCO₃; and 100 mM NaCl. The oxygenase assay medium contained 25 mM N,N-bis(2-hydroxyethyl)glycine, pH 8.2; 5 mM MgCl₂; 20 mM RuBP; and 0.5 mM of enzyme. Dissolved CO₂ was eliminated from the assay medium by adjusting the pH with carbonate-free NaOH (J. T. Baker Chemical Co., Phillipsburg, NJ) and refluxing for 30 minutes. During this time, and until it was used, "CO₂-free" air was bubbled through the assay medium. CO₂-free air was obtained by pumping air through an aspirator (VWR, San Francisco, CA) column. The zonal purification of the enzyme was dissolved in a low salt buffer (Buffer B of Chan et al. (15)). Enzyme solutions were then dialyzed against the activation medium for 12 h at 4°C. The heat-activated (50°C, 20 min) enzyme (0.05 mg) was added to 1.0 ml of CO₂-free assay medium and allowed to equilibrate for 1 min at 25°C. After addition of 10 µl of RuBP, the reaction rate was measured polarographically for 1 min. An enzyme with a specific activity of 1.0 µmol of O₂/mg of protein/min will consume oxygen at a rate of 0.4 pmol/min if the initial oxygen concentration in the assay is 250 µM.

Metal Analysis

Copper and iron content of crystallized and zonal centrifuge-purified carboxylase were measured by the method of standard additions with a Varian AA atomic absorption spectrophotometer at the University Analytical Center, University of Arizona. Metal content of all solutions with which the enzyme came into contact were also determined. Each sample was measured in both the absence and the presence of a known amount of copper and iron. The sum total of instrumental error and variation between these measurements was ±5% for observed values greater than 0.1 µg/ml of metal and ±10% for values less than 0.1 µg/ml. One-half to 1.0 ml of protein (8 to 15 mg/ml) in oxygenase activation buffer was used for each measurement. All samples had been assayed for carboxylase and oxygenase activities just prior to metal determination. Acid-washed glassware was used for preparing all assay solutions and for manipulating and storing the enzymes following zonal centrifugation. There was no difference in metal content between native and acid-hydrolyzed protein samples. The limit of detection for copper and iron was 0.003 (±0.1%) µg/ml.

X-Ray Diffraction

X-ray photographs were taken on an Elliott rotating anode generator run at 40 kV, 40 mA, with filtered Cu,Kα radiation.

RESULTS

Purification of Carboxylase

Carboxylase is readily purified in gram quantities from potato, tomato, tobacco, alfalfa, and corn by the protocol previously described (8). Cotton carboxylase is also readily purified if a 0.05 M borate buffer is used during extraction. Presumably, the borate forms a complex with gossypol (16, 17), an abundant phenolic compound in cotton leaves, and inhibits the effects of phenolic oxidation which seems to occur when the usual Tris buffer is employed for extraction. Also, apparently aiding the removal of gossypol and stabilizing the cotton enzyme is the precipitation of the crude homogenate by 30% ammonium sulfate prior to centrifugal clarification. As in the case of spinach (8), the zonal centrifuge-purified (18 S) enzyme extracted from each of these species is about 95% pure, as estimated from sodium dodecyl sulfate-acrylamide gel electrophoresis (data not shown), and possesses both carboxylase and oxygenase activities. The entire purification, excluding crystallization, requires about 48 h.

Factors Affecting Crystallization

Crystals grown by near equilibrium vapor diffusion (Methods 1, 1a, and 2) usually reach full size within 2 weeks and are often as large as 50 to 500 µm. When attempting to crystallize carboxylase from a new species, we achieved success most readily by using Method 1 (vapor diffusion with 8% PEG 6000) or Method 2 (vapor diffusion with 0.375 M ammonium sulfate). We also examined PEGs of other molecular weight ranges (600, 1,000, 4,000, and 20,000) as possible precipitants, but such substitutions produced protein precipitate and microcrystals in most cases. One exception is that PEG 4000 is effective for crystallization of tomato carboxylase (18).

A critical variable in growing crystals is pH. The pH of the initial enzyme solutions can be varied over the limited range of 6.7 to 7.2, but if carboxylase is stored in potassium phosphate buffers below pH 6.7 it cannot subsequently be crystallized. Upon mixing equal volumes of precipitant (at pH 5.4) and protein (at pH 7.2), a solution of pH 6.7 is obtained. A mixed solution of pH 6.7 yielded the largest crystals. How-
Properties of Crystals Obtained by Vapor Diffusion with PEG 6000 as Precipitant (Methods 1 and 1a)

Methods 1 and 1a yielded carboxylase crystals from alfalfa, tomato, corn, spinach, cotton, potato, and tobacco (Fig. 1). The crystal habits and the special conditions required for crystallization are recorded in Table I. Details are as follows.

Alfalfa—Alfalfa carboxylase crystals grow as rosettes of plates (Fig. 1A) at 25°C using near equilibrium vapor diffusion (Method 1). They reach 50 to 200 μm in length after 2 to 3 weeks and are the largest of the crystals grown by Method 1. The crystal plates often seem to emanate from a nuclear crystal of undetermined habit. The pH of the mixed solution from which alfalfa carboxylase is crystallized is 6.65 to 7.2, corresponding to a pH of the precipitant of 4.8 to 7.2.

Tomato—Two crystal habits (Table I, Fig. 1, B and C) of tomato carboxylase are obtained after 2 weeks by Method 1. Elongated, rodlke crystals (Fig. 1B) are obtained at 25°C using 8% PEG 6000 solutions with an initial pH of 4.8 to 7.0, corresponding to a pH of the mixed solution of 6.6 to 7.0. Flat plates (Fig. 1C) grow to 50 to 100 μm in their largest dimension with PEG 6000 at a pH of 4.8 to 6.8 as used as precipitant. With PEG 6000, microcrystals and precipitate are also present. Using a different buffer (0.025 M Tris, 5 × 10⁻⁴ M EDTA, pH 7.4) and 4% PEG 4000 as precipitant, crystals of tomato carboxylase with a hexagonal stellate habit were previously obtained (18).

Corn—Zonal-purified carboxylase from corn, a C₄ plant, crystallizes (Table I, Fig. 1D) in low yield by Method 1. These are single plates and aggregates of small platelets (5 to 20 μm), reminiscent of the alfalfa carboxylase crystals. Crystals grow at 4°C, using 8% PEG 6000 precipitants with pH values ranging from 5.4 to 6.2.

Spinach—As reported previously (8), spinach carboxylase crystals grow at 4°C, using a variation of Method 1 in which the enzyme in 0.05 M potassium phosphate at pH 7.2 is mixed with an equal volume of 8% PEG 6000 in either H₂O or 0.05 M potassium phosphate adjusted to pH values within the range 4.8 to 7.2. We have found more recently that the optimum pH of the PEG solution is 5.4, corresponding to a mixed solution at pH 6.7. Tetragonal bipyramids and other crystal habits as described previously (Ref. 8, Fig. 1E), which range in size from 5 to 50 μm, are found after 2 to 3 weeks of growth.

Cotton—Conditions for crystallization of cotton carboxylase by Method 1 are identical to those of spinach (Table I). The predominant habit of cotton carboxylase crystals is also the tetragonal bipyramid (Fig. 1F) and their size is comparable to that of the spinach crystals. However, whereas more than 80% of the spinach enzyme is present as large crystals, only a small amount of the cotton enzyme forms tetragonal bipyramids; instead, much of the protein is precipitated or is present as microcrystals.

Potato—Potato carboxylase requires the addition of 0.01 M NaCl to Buffer A to prevent precipitation in the early stages of crystallization (Method 1a). Crystals are single, flat plates, 5 to 20 μm in the largest dimension (Fig. 1G). Crystallization is effective only at 4°C, with 8% PEG 6000 in 0.05 M potassium phosphate, pH 6.2, corresponding to a pH of the mixed solution of 6.8. The yield of crystals by this method is low and variable, with most of the protein appearing as a fine amorphous precipitate.

Tobacco—Tobacco carboxylase, like that of potato, requires NaCl for crystallization (Method 1a). Unlike potato, however, crystalline tobacco carboxylase is obtained (Table I) at room temperature using 8% PEG 6000 in 0.05 M potassium phosphate with 0.01 M NaCl, over a pH range from 4.8 to 7.0 (corresponding to a pH of 6.65 to 7.0 for the mixed solution). Precipitation of enzyme during crystallization does not occur and most of the enzyme is found as single, flat plates, 50 to 100 μm in length (Fig. 1H).

Properties of Crystals Obtained with Ammonium Sulfate as Precipitant by Methods 2 and 4

Method 2 is a variation of Method 1 in which 0.375 M ammonium sulfate is substituted for 8% PEG 6000. To date, this method has yielded crystals of carboxylase from potato, tobacco, and tomato, but has been ineffective with the enzyme from other species. Method 4 uses the same solutions as does Method 2, but the solutions are mixed by dialysis rather than by rapid mixing followed by vapor diffusion.

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Potato and tobacco—Method 2 produces crystals of potato and tobacco carboxylase in 1 to 2 weeks at 25°C (Table II). The potato crystals are tetragonal bipyramids, resembling the tobacco crystals grown by dialysis in 0.4 M potassium phos-
Crystallization of RuBisCO

**Table II**

<table>
<thead>
<tr>
<th>Species</th>
<th>Crystal habit</th>
<th>Temperature</th>
<th>NaCl concentration</th>
<th>pH of precipitant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato</td>
<td>Tetragonal bipyrad</td>
<td>22-25</td>
<td>10 mM</td>
<td>4.8-6.2</td>
</tr>
<tr>
<td>Tobacco</td>
<td>Rhombic decahedron</td>
<td>22-25</td>
<td>10 mM</td>
<td>5.4</td>
</tr>
<tr>
<td>Tomato</td>
<td>Stellate regular hexahedron</td>
<td>22-25</td>
<td></td>
<td>4.8-7.0</td>
</tr>
</tbody>
</table>

The crystals of potato and tobacco are large (100 to 600 μm) and mechanically stable (Fig. 1). Large crystals of tobacco enzyme have been grown from both the species *Nicotiana tabacum* and *Nicotiana sylvestris*. Crystals from tobacco have been obtained with the ammonium sulfate/Buffer B solution at pH 5.4, and potato crystals were obtained at pH values ranging from 4.8 to 6.2 (corresponding to pH values of the mixed solution of pH 6.6 to 6.8).

**Preparative Scale Crystallization (Methods 3 and 4)**

Large quantities of crystals (about 70% recovery) of alfalfa, tobacco, cotton, and spinach carboxylase were obtained by preparative vapor diffusion (Method 3). In all instances, the crystal habit is that obtained by near equilibrium vapor diffusion, although the crystals are less regular and rarely are larger than about one-third of the largest crystals obtained by Method 1.

Method 4 is useful for preparative crystallization of tobacco and potato carboxylase. A small amount of precipitate is found with the potato crystals but is absent from the tobacco carboxylase crystallizations. Crystals of potato carboxylase are smaller than those grown by Method 1a, but are identical in habit to them. Large tobacco carboxylase crystals have been grown by this method.

**Carboxylase and Oxygenase Activities**

The catalytic activities of alfalfa, spinach, tobacco, and tomato carboxylase were measured before and after crystallization. Carboxylase enzyme from each of these species has both carboxylase and oxygenase activities (Table III) which are stable for at least several weeks in the case of spinach and alfalfa. Crystallization by any of the methods used produced no significant change in specific activities of the protein from various species examined. The catalytic activities of the enzyme of all plants examined was low, relative to spinach.

To investigate possible effects of precipitants, we measured the activities of tobacco and tomato enzyme, after crystallization by various means. We found no such effect, as shown in Table III. PEG 6000, added directly to the carboxylase reaction mixture, reduces the activity to 80 to 90% of that obtained in the absence of PEG. Ammonium sulfate is a competitive inhibitor with respect to RuBP (19) in the carboxylase reaction and must be completely removed from the dissolved crystals by dialysis before assay.

**Metal Content of Crystals**

The copper and iron contents of crystalline and zonal centrifugation-purified carboxylase from alfalfa, spinach, tobacco,

<table>
<thead>
<tr>
<th>Species</th>
<th>Carboxylase activity</th>
<th>Oxygenase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinach</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>11 0.85(±0.07)</td>
<td>8 0.08(±0.01)</td>
</tr>
<tr>
<td>After</td>
<td>11 0.94(±0.08)</td>
<td>8 0.08(±0.02)</td>
</tr>
<tr>
<td>Tobacco</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>6 0.19(±0.02)</td>
<td>5 0.02(±0.01)</td>
</tr>
<tr>
<td>After</td>
<td>6 0.19(±0.02)</td>
<td>5 0.02(±0.01)</td>
</tr>
<tr>
<td>Cotton</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>3 0.08(±0.01)</td>
<td>4 0.010(±0.001)</td>
</tr>
<tr>
<td>After</td>
<td>3 0.08(±0.01)</td>
<td>4 0.010(±0.004)</td>
</tr>
<tr>
<td>Potato</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>7 0.18(±0.01)</td>
<td>8 0.03(±0.01)</td>
</tr>
<tr>
<td>After</td>
<td>7 0.22(±0.02)</td>
<td>8 0.03(±0.01)</td>
</tr>
<tr>
<td>Tomato</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>3 0.08(±0.01)</td>
<td>4 0.020(±0.004)</td>
</tr>
<tr>
<td>After</td>
<td>3 0.08(±0.01)</td>
<td>4 0.020(±0.003)</td>
</tr>
</tbody>
</table>

* Values are the mean ± SEM of three determinations.

**X-Ray Diffraction**

Crystals of potato and tobacco carboxylase, grown by Methods 2 and 4, respectively, were studied by x-ray diffraction. X-ray precession photographs were recorded by standard methods. The hk0 zones are shown in Fig. 2 for crystals of *N. tabacum* (reported earlier in Ref. 7), *N. sylvestris*, and potato.

The intensity distributions of all three x-ray photographs are essentially the same, although the potato photograph exhibits the diffuseness in the higher angle reflections that is typical of older carboxylase crystals that are not stored under nitrogen. The similarity of these x-ray photographs suggests that the contents of the three unit cells are closely similar. Furthermore, measurements of the unit cell dimensions of the photographs of the crystals from *N. sylvestris* and potato shows that they are identical to the Form III crystals of *N. tabacum*. This is shown in Table V. We were able also to establish that the space group of the crystals from *N. sylvestris* is the same as that from *N. tabacum*. Only an hk0 zone was available for the potato carboxylase crystal, and this photograph is consistent with the same space group. Quantitative

Crystallization of RuBisCO

TABLE IV
Copper and iron content of RuBP carboxylase/oxygenase before and after crystallization

<table>
<thead>
<tr>
<th>Species</th>
<th>Copper</th>
<th>Iron</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td></td>
<td>g atom/mol protein</td>
<td>g atom/mol protein</td>
</tr>
<tr>
<td>Spinach</td>
<td>0.05±0.01&quot;*&quot;</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>Tobacco</td>
<td>0.07±0.01</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>Method 3 crystals</td>
<td>0.07±0.01</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>Low salt dialysis</td>
<td>0.07±0.01</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>Alfalfa</td>
<td>0.22±0.01</td>
<td>0.13±0.01</td>
</tr>
<tr>
<td>Tomato</td>
<td>0.11±0.01</td>
<td>0.09±0.01</td>
</tr>
<tr>
<td>Method 3 crystals</td>
<td>0.43±0.02</td>
<td>0.40±0.02</td>
</tr>
<tr>
<td>Method 4 crystals</td>
<td>0.43±0.02</td>
<td>0.18±0.02</td>
</tr>
</tbody>
</table>

* Uncertainties represent the maximum deviation from the mean of the measurements.
* Data from Johal and Bourque (8).

Fig. 2. X-ray precession photographs for the hk0 zone, recorded from crystals of carboxylase from three plant sources. a, N. tabacum carboxylase, Form III; precession angle $\bar{\mu} = 10^\circ$. b, N. sylvestris carboxylase; $\bar{\mu} = 10^\circ$. c, Carboxylase from potato; $\bar{\mu} = 8^\circ$. 
produce crystals in any instance. Our detailed results demonstrate that it may be necessary to vary the parameters of crystallization somewhat from species to species. For example, 10 mM NaCl must be added to crystallize potato or tobacco carboxylase, and the temperature must be maintained at room temperature (for crystallization of alfalfa, tomato, and tobacco enzyme) or 4°C (for crystallization of corn, spinach, cotton, and potato carboxylase). The second set of conditions are those of Method 2, in which ammonium sulfate replaces PEG 6000 as the precipitant. These conditions also must be varied somewhat for optimum crystal growth (Table II).

Several different proteins have previously been crystallized by vapor diffusion using PEG as a precipitant (21). However, the tendency of carboxylase from a variety of species to crystallize under similar conditions in our laboratory is somewhat atypical of proteins. For example, McPherson (22), in his review of protein crystal growth, states, “There is often a wide variability in the ease and quality with which proteins and nucleic acids can be crystallized in going from species to species . . . Presumably because the lattice forces rely on so few contacts between molecules, only very minor changes in surface charge or residue disposition may have a profound effect on the macromolecular interactions.” The converse of this presumption is that the crystallization of several carboxylases under similar conditions suggests that the surface charges and residue disposition of the molecule have been conserved in this enzyme to an unusual extent. Indeed, the isoelectric points of tobacco, spinach, cotton, and corn carboxylase are almost identical in the presence of the activators, MgCl₂ and NaHCO₃, or the substrate, RuBP (23). In addition, crystals of identical habit (presumably Form I) have been obtained from plants of different genera (various Nicotiana species and eggplant, Solanum melongena, (20, 24)). These observations lead to the question of why the molecular surface of carboxylase might be conserved among plant species.

Before speculating on this question, we must ask whether comparison of the intensities of diffraction from the photographs of the two tobacco species confirms that the diffraction patterns are identical within experimental error.

Thus, methods of x-ray diffraction establish that the crystals of carboxylase from potato and tobacco grown by Methods 2 and 4 have the same crystal structure as the Form III crystals of N. tabacum, grown by equilibrium dialysis (7).

**DISCUSSION**

**Purification**—We have found that the purification procedure previously reported (8) for spinach carboxylase is also effective for isolation of enzyme from tobacco, tomato, potato, corn, and alfalfa. For cotton, a modified procedure is required that uses borate buffer; it is recommended for other plant species in which polyphenols and terpenoids are particularly abundant. We find that carboxylase purified by either method yields crystals by the methods described here. The presence of NaCl during homogenization is unnecessary for some species, e.g. cotton and alfalfa. However, in the absence of an established purification protocol, the addition of 0.2 M NaCl is advisable. Its presence during purification has no apparent effect on carboxylase crystallization or enzyme activity and may be required to prevent precipitation, as in the case of potato and tobacco.

**Crystallization**—Carboxylase from many species of Nicotiana crystallizes (as Form I) under identical low salt dialysis conditions (20). We have extended these results by finding that carboxylase isolated from several taxonomically diverse species crystallizes under similar conditions. Two sets of solution conditions yield macroscopic crystals of carboxylase from seven different plants. The conditions of Method 1 have produced crystals from seven species and have not failed to produce crystals in any instance. Our detailed results demonstrate that it may be necessary to vary the parameters of crystallization somewhat from species to species. For example, 10 mM NaCl must be added to crystallize potato or tobacco carboxylase, and the temperature must be maintained at room temperature (for crystallization of alfalfa, tomato, and tobacco enzyme) or 4°C (for crystallization of corn, spinach, cotton, and potato carboxylase). The second set of conditions are those of Method 2, in which ammonium sulfate replaces PEG 6000 as the precipitant. These conditions also must be varied somewhat for optimum crystal growth (Table II).

Several different proteins have previously been crystallized by vapor diffusion using PEG as a precipitant (21). However, the tendency of carboxylase from a variety of species to crystallize under similar conditions in our laboratory is somewhat atypical of proteins. For example, McPherson (22), in his review of protein crystal growth, states, “There is often a wide variability in the ease and quality with which proteins and nucleic acids can be crystallized in going from species to species . . . Presumably because the lattice forces rely on so few contacts between molecules, only very minor changes in surface charge or residue disposition may have a profound effect on the macromolecular interactions.” The converse of this presumption is that the crystallization of several carboxylases under similar conditions suggests that the surface charges and residue disposition of the molecule have been conserved in this enzyme to an unusual extent. Indeed, the isoelectric points of tobacco, spinach, cotton, and corn carboxylase are almost identical in the presence of the activators, MgCl₂ and NaHCO₃, or the substrate, RuBP (23). In addition, crystals of identical habit (presumably Form I) have been obtained from plants of different genera (various Nicotiana species and eggplant, Solanum melongena, (20, 24)). These observations lead to the question of why the molecular surface of carboxylase might be conserved among plant species.

Before speculating on this question, we must ask whether

---

**Table V**

<table>
<thead>
<tr>
<th>Species</th>
<th>Crystal form</th>
<th>Reference</th>
<th>Space group</th>
<th>a (Å)</th>
<th>c (Å)</th>
<th>Number of molecules/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. tabacum</td>
<td>I</td>
<td>5</td>
<td>P4₁2₂</td>
<td>14.32</td>
<td>383 ± 3</td>
<td>12</td>
</tr>
<tr>
<td>N. tabacum</td>
<td>II</td>
<td>6</td>
<td>P4₁2₂</td>
<td>230 ± 2</td>
<td>383 ± 3</td>
<td>6</td>
</tr>
<tr>
<td>N. tabacum</td>
<td>III</td>
<td>7</td>
<td>I422</td>
<td>148.7 ± 0.2</td>
<td>315 ± 3</td>
<td>2</td>
</tr>
<tr>
<td>N. sylvestris</td>
<td>III</td>
<td>This paper</td>
<td>I422</td>
<td>148.7 ± 0.2</td>
<td>137.5 ± 0.2</td>
<td>2</td>
</tr>
<tr>
<td>Potato</td>
<td>III</td>
<td>This paper</td>
<td>(I422)</td>
<td>148.4 ± 0.6</td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

---

**Table VI**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Crystal form</th>
<th>Morphology</th>
<th>Method</th>
<th>Protein solution</th>
<th>Precipitant</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Form I</td>
<td>Rhombic dodecahedrons</td>
<td>Dialysis</td>
<td>24 mM Tris, pH 7.4-8.8</td>
<td>24 mM Tris, pH 7.4-8.8</td>
</tr>
<tr>
<td>6</td>
<td>Form II</td>
<td>Square plates, triangular prisms</td>
<td>Dialysis</td>
<td>24 mM Tris, pH 7.4</td>
<td>50 mM potassium phosphate, pH 6</td>
</tr>
<tr>
<td>3</td>
<td>Form II</td>
<td>Mixing with precipitant followed by vapor diffusion</td>
<td></td>
<td>20 mM NaCl/Tris buffer, pH 7.8, 70 mM NaCl</td>
<td>4% PEG 6000</td>
</tr>
<tr>
<td>7</td>
<td>Form III</td>
<td>Truncated tetragonal bipyramids</td>
<td>Dialysis</td>
<td>50 mM potassium phosphate, pH 7.2</td>
<td>300 mM (NH₄)₂SO₄ in 200 mM potassium phosphate, pH 5.2</td>
</tr>
<tr>
<td>This paper</td>
<td>Form III</td>
<td>Tetragonal bipyramids</td>
<td>Methods 2 and 4</td>
<td>50 mM potassium phosphate, pH 7.2, (10 mM NaCl)²</td>
<td>375 mM (NH₄)₂SO₄ in 50 mM KH₂PO₄, pH 5.4</td>
</tr>
<tr>
<td>This paper</td>
<td>Unknown, possibly mixed</td>
<td>Varied²</td>
<td>Methods 1 and 1a</td>
<td>50 mM potassium phosphate, pH 7.2, (10 mM NaCl)²</td>
<td>8% PEG 6000 in 50 mM KH₂PO₄, pH 5.4</td>
</tr>
</tbody>
</table>

² Depending on species, see Tables I and II.
all the various crystals observed by us are of the same space

group and cell dimensions or, in other words, if they are the

same crystal form. A definitive answer can be given only for

these forms examined by x-ray diffraction. These are the

crystals of potato grown by Method 2 and the two species of

tobacco grown by the related Method 4. These three forms all

have the packing of the Form III crystal, grown previously by

slow dialysis (see Table VI). Though the earlier method mixes

the low pH ammonium sulfate solution with the protein far

more slowly than the present Method 2, the solutions being

mixed are closely similar (compare Lines 4 and 5 of Table VI).

This suggests that Form III crystals are nucleated by exposing

the protein to a solution of pH near 5, and that the nuclei

grow into crystals either after the pH is raised to 6.7 (as in

Method 2) or if it remains at about 5.4 (as in Method 4 and

Ref. 7).

What form are the crystals grown by Methods 1 and 1a? In

these methods, as in Method 2, there is a nucleation step at

about pH 5.4, but the precipitant is now 8% PEG. Because of

the proposed low pH nucleation, we might suspect that these

crystals are also Form III. In fact, the crystals of spinach

cotton in Fig. 1E and F appear virtually identical in their
tetragonal bipyramidal habit to the crystals of potato (Fig. 1)

and to crystals of N. tabacum grown at pH 5, both known to

be Form III. In contrast, the other crystals grown by Methods

1 and 1a have a flat plate habit, more reminiscent of Form II

crystals (Line 2 of Table VI). However, crystal habit reflects

rates of growth more than it reflects internal structure. In

short, at least two of the four crystals grown by Method 2 are

Form III, and the crystals grown by Methods 1 and 1a may be

Form III, but there is no firm evidence.

An interesting contrast is presented by the comprehensive

classification of hemoglobin crystals by Reichert and Brown

(25). Working before the development of x-ray crystallogra-

phy, they characterized hemoglobin crystals from the blood

of over 100 species. They found the crystals from any genus to

be "isomorphous," but the crystals of different genera were

distinct, except for closely related genera like dogs and foxes.

Our findings of identical crystal forms from the two species of

tobacco thus fits their rule, but our discovery that potato and

tobacco yield the same crystal form is an exception.

Regarding the reasons why carboxylase from taxonomically

diverse plants tends to crystallize under similar conditions,

the possibility that the disposition of some residues on the

molecular surface is conserved among these species prompts

us to question why the surface of carboxylases should be

conserved more strongly than those of other proteins, which
do not usually crystallize from similar solutions. The answer

must be that some surface residues are adapted to crucial

function(s) which may be disrupted by any change. This

function may be related to regulation of enzymatic activity or
to the propensity of the protein to crystallize in the Form III

structure. The reason for thinking that crystallization may be

an important function of this enzyme is that there is evidence

that the crystalline inclusions found in chloroplasts may be

the carboxylase (11, 12).

Specific Activities and Metal Contents—The availability of

crystalline enzyme from several species has allowed us to
determine the activities of the protein's oxygenase and

carboxylase functions and the copper and iron contents. The

ratio of the two activities is clearly similar among the various

species and does not change as a result of crystallization

(Table III). This tends to confirm that the two activities are

invariably linked, as opposed to separable, as claimed by

Branden (26) for parsley. Branden's results have not been

obtained with parsley enzyme in other laboratories (27).

Carboxylase activities of tobacco, alfalfa, and potato enzyme

are similar within experimental error, whereas the activities

of the cotton and tomato enzymes were about half that of

potato enzyme. RuBP oxygenase activities of all these en-

zymes were similar. However, both enzymatic activities of

the spinach enzyme were several times greater than that of

the majority of the other species examined. This suggests that

spinach carboxylase is inherently more active than that of

other species. No attempt was made to tailor the purification

scheme with regard to enzyme activity or to optimize the

carboxylase or oxygenase assay and activation conditions for

these species. Thus, the conditions used were those optimal

for spinach carboxylase (8, 14) but not necessarily optimal for

the enzyme from other species. Further work will be necessary
to establish whether the spinach activities are intrinsically
greater than the maximal attainable activities of the other

carboxylases. However, it is likely that variable growth con-
ditions and physiological status of the leaf tissue among these

species are the major determinants of the low specific activi-
ties we observed. Variable levels of polyphenols were present

in the leaves of several species as indicated by different
degrees of browning coloration in the leaf homogenates. Per-

haps significant protease activities, which might be present in

the homogenates from some of the species examined, may also

contribute to the low specific activities observed.

Our rationale for measuring metal contents was the earlier

investigation reports (19, 28, 29) that chromatographically

purified spinach enzyme contains 1.0 g atom of copper/mol,

that some monooxygenases have iron as a cofactor, and that

the role of metals in catalysis of this enzyme's reactions has

been controversial and as yet unresolved (30, 31). On the

average, we find less than 0.2 g atom per mol of protein of
either metal in the crystalline enzyme. Furthermore, copper

and iron are each present in our oxygenase assay solutions in

amounts less than 0.2 g atom per mol of enzyme used for the

assay. Thus, at most, there would have been 0.4 g atom per

mol of protein present during the oxygenase reaction. These

results suggest that stoichiometric amounts of these metals

are neither tightly bound to the crystalline enzyme nor are

they required as cofactor for the oxygenase reaction. It is,

however, possible that only a small fraction of the enzyme

molecules are active and that all of the copper (or iron)
present is associated with these molecules during catalysis.

Homogeneous, crystallized carboxylase may be another ex-

ample of an enzyme which possesses "active site heterogene-

ity" (32) and which has as yet undefined prosthetic metal

stoichiometry (33). Regarding this point, measurements were

made of the prosthetic copper content and enzyme activity of

167 preparations of ascorbate oxidase over a period of 23 years

(32). The data show that, as purification methods improved,

the activity per µg of copper of homogeneous enzyme prepa-

rations remained relatively constant while the copper content

on a protein basis doubled during this period. Thus, the

specific activity (units/mg of protein) of ascorbate oxidase

increased as a function of the copper content. Since our

procedure for carboxylase purification utilizes EDTA-contain-

ing buffers, it is possible that bona fide prosthetic copper (or

iron) may be lost during protein purification. However, addi-
tion of copper to purified enzyme does not increase its specific

oxygenase activity (28, 30, 31). Further examinations of the

metal content of the enzyme, purified in the absence of che-
lating agents, may clarify these issues.

We conclude that crystalline carboxylase from a variety of

plants is readily available for comparative chemical and phis-

ical characterizations. We have initiated such studies with

measurements of enzyme activities and metal contents. Struc-

"J. T. Bahr, M. S. Capel, S. Johal, and D. P. Bourque, unpublished

results.
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