The Effect of Greening of Sorghum Leaves on the Molecular Weight of a Complex Containing 4-Hydroxyacinnamic Acid Hydroxylase Activity

H. A. Stafford and M. Bliss
Department of Biology, Reed College, Portland, Oregon 97202

ABSTRACT

During the greening of leaves of Sorghum bicolor var. Wheatland milo, the activity of 4-hydroxyacinnamic (p-coumaric) acid hydroxylase in pH 6 buffered extracts was shifted from a relatively low to a high molecular weight fraction. Differences between these forms found in etiolated and green leaves were based on differential centrifugation, ammonium sulfate precipitation, and on elution patterns from Agarose A-15m. Both molecular weight forms were precipitated by protamine sulfate at pH 6, and approximately 40 to 80% of the activity of each form was associated with a 500 to 37,000g pellet when tissues were ground at pH 8 in media of either high or low osmotic concentration. Although no fraction with hydroxylase activity was ever found without any chlorogenic acid oxidase activity, the two activities frequently varied independently, and could be partially separated from each other, using the above techniques. Comparisons were made with the very small molecular weight form of 4-hydroxyacinnamic acid hydroxylase characteristic of tissues of first internodes. The significance of these results in terms of possible multienzyme complexes capable of converting phylal alanine and tyrosine to cinnamic acid derivatives is discussed.

The localization of polyphenoloxidase in higher plants, including its monophenol hydroxylating activity, continues to be controversial, the activity being either easily solubilized or bound to membranes of chloroplasts and other organelles (2, 4, 7, 15, 17, 18, 20, 22-26). Recently, the possibility that a flavonoid is an electron acceptor in photosynthesis (14), and the demonstration of a CoA-dependent synthesis of a benzene nucleus in chloroplasts which may represent the site of synthesis of the A-ring of flavonoids (10), add to the interest in a chloroplast localization for the hydroxylase. Furthermore, the known electron donors for the hydroxylation of 4-hydroxyacinnamic acid, ascorbate, NADPH, and reduced pteridine derivatives (20, 26) are produced in chloroplasts (13, 25). Recent studies (20) demonstrating the association of the HCH activity with a high molecular weight complex have been extended to include the effect of greening of etiolated leaves of sorghum on this complex.

MATERIALS AND METHODS

Seeds of Sorghum vulgaris var. Wheatland milo used in previous studies (19-21) were grown either on moist filter paper in closed trays at 25 C for seedling stages or in pots with Gro-tron as soil in a growth chamber at about 30 C for leaf tissue. Green shoots grew better and the enzymatic activities were greater if grown at 30 C rather than 20 C. White light at about 1400 ft-c was given in a 16-hr photoperiod.

Etiolated leaves were used from either completely dark-grown shoots, in which the primary leaf was only partially developed by 7 days, due to the prior growth of the first internode (mesocotyl), or after the first internode was limited to about 1 cm in length by 6 hr of bright light at 2 days of germination in the dark. This light pretreatment at the time that the coleoptiles were just showing above the soil surface permitted the subsequent growth of etiolated second and third leaves. Since primary leaves showed some effects from the early light treatment (production of small amounts of a cyanidin), they were generally discarded.

Unless specified otherwise, tissues were ground in a mortar twice their fresh weight in 50 mm MES buffer at pH 6 in the presence of sand and Polyclar AT (10% of the fresh weight of the tissue). Phosphate buffers were used for a pH of 7.4, while 50 mm Bicine and Tricine buffers were used for pH 8. At pH 8, Dowex 1-X8, Cl⁻ form, 200 to 400 mesh, at 20% of the fresh weight of tissues was substituted for Polyclar AT. After cheesecloth filtration and centrifugation at 500g for 1 min to remove the Polyclar, the supernatant fraction was centrifuged for 15 min at 37,000g, removing a crude particulate pellet containing chloroplasts (P₁). The subsequent supernatant fraction was then precipitated with ammonium sulfate added as a solid in either a broad cut (0-500 g/l) or in two narrower cuts at 0 to 200 and 200 to 500 g/l and centrifuged after sitting for 30 min at 4 C. Pellets were generally suspended in 50 mm MES at 10% of the original volume.

Gel Filtration Column Chromatography. Agarose (Bio-Gel A-15m, 100-200 mesh) was equilibrated with 100 mm phosphate buffer at pH 6 in columns (1 x 40 cm). Generally 1 ml of an ammonium sulfate concentrate was added to the agarose columns. One-ml fractions were collected at a rate of approxi-

---

1 This work was supported by Grant GB 28597X from The National Science Foundation.

2 Abbreviations: HCH: 4-hydroxyacinnamic acid (p-coumaric acid) hydroxylase; CAO: chlorogenic acid oxidase; CA: caffeic (3,4-dihydroxyacinnamic) acid.

---

3 Cultivated sorghums are now called Sorghum bicolor (Linn) Moench. See ref (5), pp. 27-28.
Table I. Ammonium Sulfate Fractionation of HCH and CAO Activities in Green Shoots

Tissues were extracted in 50 mm MES at pH 6. The 37,000g pellet which contained less than 2% of the total activity was discarded prior to the ammonium sulfate precipitation.

<table>
<thead>
<tr>
<th>Ammonium sulfate (g/l)</th>
<th>HCH (%)</th>
<th>CAO (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-50</td>
<td>22</td>
<td>5</td>
</tr>
<tr>
<td>50-100</td>
<td>38</td>
<td>25</td>
</tr>
<tr>
<td>100-200</td>
<td>40</td>
<td>25</td>
</tr>
<tr>
<td>200-500</td>
<td>&lt;1</td>
<td>44</td>
</tr>
<tr>
<td>Total activity/g fresh wt</td>
<td>1.8 Slope 360</td>
<td></td>
</tr>
</tbody>
</table>

chlorotically 3 min/ml. Individual fractions were assayed or were combined into four groups called fractions I to IV as indicated in Figures 1 and 2, and were concentrated by precipitation with 500 g/l ammonium sulfate. Spot tests for CAO and peroxidase (20, 21), as well as visual estimates of cloudiness and color, were used as markers.

Enzyme Assays. HCH was assayed in a 1-ml volume containing 4 μmol of hydroxycinnamate, 4 μmol of ascorbate, 0.1% bovine serum albumin in 50 mm MES, pH 6, generally at two enzyme concentration levels. The reaction was started by the addition of enzyme. One- and 3-hr incubation times at 30 C with shaking were used for leaf and first internode extracts, respectively, the reaction being linear during this period. The reaction was stopped with 0.03 ml of 6 N HCl. A 0.1-ml aliquot was chromatographed with caffeic acid standards in a benzene-acetic acid-water mixture (40:15:1, v/v). The blue fluorescing spots corresponding to caffeic acid were eluted in methanol and aliquots were assayed fluorimetrically (19, 20).

CAO was measured at 30 C with an oxygen electrode (20). Results are recorded as slopes per g fresh weight of original tissue in a 2-ml volume and can be converted to μmol O_2/hr·g fresh weight by multiplying this value by 0.924.

RESULTS

Activities in Dilute Buffer Extracts at pH 6. In extracts of green shoots grown in 50 mm MES or phosphate buffers, most of the activity for both HCH and CAO was associated with the supernatant fraction after centrifugation at 37,000g. Approximately 45% of the total activity, but only 19% of the CAO, was associated with a microsomal fraction precipitated at 100,000g. In the rest of the work described in this paper, this microsomal fraction was generally not separated from the supernatant fraction after centrifugation at 37,000g.

Ammonium Sulfate Precipitation. In extracts of green shoots most of the HCH activity, but only about 55% of the CAO activity, was precipitated by ammonium sulfate at 200 g/l (Table I). However, most of the HCH activity isolated from tissues of first internodes, etiolated coleoptiles and leaves were precipitated only above 200 g/l (Table II). After 6 hr of greening, the percentage associated with the 0- to 200-g fraction increased. Mixing experiments in which etiolated and green leaves were ground together indicated that these differences with greening of the leaves were not due to alterations occurring during the extraction procedure (Table III).

Column Chromatography with Agarose A-15m. When a 0- to 200-g/l ammonium sulfate fraction from green leaves was chromatographed on an Agarose A-15m column, only about 50% of the activity was recovered. If the microsomal fraction was removed prior to ammonium sulfate precipitation, recovery was 100% or greater, presumably due to the removal of inhibitors. Approximately 90% of the activity eluted with 50 mm phosphate buffer, pH 6, was found in a peak near the void volume (fraction I), indicating the presence of a very high molecular weight complex (Fig. 1). Fragments of green membranes, present if the microsomal fraction was not removed, were also found in fraction I at the void volume as well as being trapped on the column. The remaining 10% of the activity recovered was associated with a shoulder in the region of fraction II. Fraction I probably contains particles of varied sizes, including membrane fragments that can pass through the agarose column.

In extracts of etiolated first internodes grown at 25 C and incubated as excised internodes (19), or from intact plants grown in soil at 30 C, all of the HCH activity was found in the smallest molecular weight fraction (fraction IV) (Table IV). This was where the bulk of the peroxidase isozyme C3 was found (20). A low but definite CAO activity was also detected in the same peak (slope of 0.16/g fresh weight).

Activities found in etiolated coleoptiles and primary leaves were found in all four fractions, but mostly in fraction II (Table II).
ble IV). The activity in extracts of etiolated second and third leaves was still mainly in fraction II, but was no longer detectable in fraction IV. After 6 hr of light for partial greening of the leaves, the activity recovered from the 200- to 500-g ammonium sulfate fraction was mainly in fraction II. Neither of these partially or completely greened leaves showed any activity in fraction I from the 200- to 500-g ammonium sulfate precipitate, but activity in this fraction I would have been either removed in the prior precipitation with 200 g of ammonium sulfate or would have been trapped on the column.

About 55% of the total HCH activity recovered from extracts of etiolated leaves was found in fraction II (Table IV). This may actually be a complex of peaks (Fig. 2), but it is considered here as one peak until further experiments determine whether such multiple peaks or shoulders are valid.

![Figure 1](image1.png)

**Figure 1.** Elution profiles of HCH (——) and CAO (X • • • X) activities eluted from 1 × 40 cm Agarose A-15m columns with 100 mM phosphate, pH 6. The combined fractions called fractions I to IV are shown at the top. A sharp peak of peroxidase activity according to visual spot tests was found in fraction I and a broad peak throughout fractions III and IV. Fig. 1: Activities from green leaves in the 0 to 500 g ammonium sulfate fraction, minus the 100,000g pellet. The total HCH activity recovered (158%) was equivalent to 0.54 umole caffeic acid produced/hour·g fresh weight. Peaks of CAO activity based on visual spot tests are indicated. Fig. 2: Activities from etiolated leaves in the 200- to 500-g ammonium sulfate fraction, minus the 37,000g pellet. The total HCH activities recovered (150%) was 0.18 umoles/hr·g fresh weight. See Table IV for per cent recovered in each of four peaks. Total CAO activity was equal to a slope of 240/hr·g fresh weight or 222 umoles Os/hr·g fresh weight. Approximately 10% of the activity was in fraction I, 25% in II, and 64% in III.

**Table IV. Differences in Elution Patterns of HCH Activity from Agarose A-15m Columns of Extracts from Etiolated Shoots and Etiolated and Green Leaves**

Fractions in the areas I to IV shown in Figs. 1 and 2 were pooled for analysis or the activities of separate fractions were summed. All extracts were made as in Table I.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Activity in Fraction I</th>
<th>Activity in Fraction II</th>
<th>Activity in Fraction III</th>
<th>Activity in Fraction IV</th>
<th>Total Activity Recovered (μmoles CA/g fresh wt)</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-200 g ammonium sulfate/1</td>
<td>90 10 0 0</td>
<td>20-500 g ammonium sulfate/1</td>
<td>First internodes</td>
<td>0 0 0 100</td>
<td>0.02</td>
<td>400</td>
</tr>
<tr>
<td>Etiolated coleoptiles + primary leaves</td>
<td>26 43 9 22</td>
<td>Etiolated leaves</td>
<td>25 55 20 0</td>
<td>0.18</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Etiolated leaves</td>
<td>6</td>
<td>0 90 10 0</td>
<td>0.10</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hr greening</td>
<td>0 0 0 0</td>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 37,000 to 100,000g pellet was removed and analyzed separately, otherwise much of this activity is trapped on the column (see text).

2 Internodes from plants grown in soil in the dark at 30 C.

3 Mainly 2nd and 3rd leaves, primary leaves were discarded. See Fig. 2 for actual elution profile for extract from etiolated leaves.

While no HCH fraction has yet been found without any CAO activity, the bulk of the CAO activity was frequently found in different peaks either on Sephadex G-100 (20) or Agarose A-15m. In green and etiolated leaves, the activity in the 200- to 500-g ammonium sulfate fraction was predominantly in fraction III (Fig. 2).

**Effect of pH and Osmotic Concentration on the Distribution of Activities in a 37,000g Pellet.** The percentage of HCH and CAO activity of green leaves in a particulate fraction precipitated between 500 and 37,000g was dependent upon the initial pH of the extract rather than upon the osmotic concentration necessary to maintain chloroplasts intact (Table V). This crude pellet was generally not washed by recentrifugation in the original medium because of inactivation, even though reactivation occurred upon freezing and thawing in the presence of substrate, a phenomenon being studied further. The pellet was resuspended in 50 mM MES buffer for assay. Results were variable, perhaps indicating the presence of inhibitors or activators. At pH 6 in either low or high osmotic concentrations, most of the activities were associated with the supernatant fraction after centrifugation at 37,000g, whereas at pH 8, in either high or low osmotic concentrations, up to 80% of the activity recovered was associated with the 37,000g pellet (P). Approximately 19% of this activity of P, was found in a 500 to 1,000g pellet, 36% at 1,000 to 20,000, and 14% at 20,000 to 37,000. This particulate activity obtained between 500 and 37,000g was partly solubilized upon resuspension in dilute buffer, and completely after treatment with 0.5% Triton X-100. However, upon chromatography on Sephadex G-100, the activity was almost entirely in the void volume fraction, an indication that the activity remained with a large complex.

There was no significant change in this pH-dependent association of activity with P, in either etiolated or partially
Table V. Distribution of HCH and CAO Activities between Particulate Fractions Obtained between 500 and 37,000 g (P1) Compared with That of the Supernatant Fraction Precipitated with Ammonium Sulfate

Ranges are given for extracts from green leaves and individual values for etiolated leaves and are expressed as percentage of total activity recovered. Tissues were ground in media of low (L) and high (H) osmotic concentrations (O.C.) and various pH values.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>O.C.</th>
<th>HCH</th>
<th>CAO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ammonium Sulfate (g/l)</td>
<td>Total Activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P1</td>
<td>0-200</td>
</tr>
<tr>
<td>Etiolated leaves</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MES</td>
<td>6</td>
<td>L</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>Tricine</td>
<td>8</td>
<td>L</td>
<td>3</td>
<td>24</td>
</tr>
<tr>
<td>Tricine</td>
<td>8</td>
<td>H</td>
<td>92</td>
<td>3</td>
</tr>
<tr>
<td>Etiolated leaves and 6 hr of greening</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MES</td>
<td>6</td>
<td>L</td>
<td>0</td>
<td>59</td>
</tr>
<tr>
<td>Tricine</td>
<td>8</td>
<td>H</td>
<td>49</td>
<td>6</td>
</tr>
<tr>
<td>Green leaves</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MES or phosphate</td>
<td>6</td>
<td>L</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>H</td>
<td>38</td>
<td>8</td>
</tr>
<tr>
<td>Phosphate</td>
<td>7.4</td>
<td>H</td>
<td>22-82</td>
<td>0.15-0.48</td>
</tr>
<tr>
<td>Bicine</td>
<td>8</td>
<td>H</td>
<td>40-80</td>
<td>0.14-0.2</td>
</tr>
</tbody>
</table>

^ Media contained either 0.5 mM sucrose with or without 3 mM MgCl2, with phosphate buffers or 0.33 mM mannitol, 2 mM KNO3, 2 mM Na2 EDTA, 2 mM ascorbate, 1 mM MnCl2, 1 mM MgCl2, 0.5 mM KH2PO4, 20 mM NaCl, plus 50 mM MES, pH 6 or Bicine pH 8 (9).

100 mM Tricine, pH 7.9, 600 mM sorbitol, 3 mM Ca(NO3)2, 0.1% Na ascorbate (10).

Phosphate buffer plus sucrose generally was inhibitory (20).

greened leaves. But pH had no effect in etiolated leaves on the association of the activity remaining in the supernatant with 200- to 500-g ammonium sulfate/fraction.

CAO activity followed a similar pattern of distribution, frequently with higher activities found at pH 8 than 6. Upon solubilization of the 37,000-g pellet with Triton X-100 and chromatography on Sephadex G-100, the bulk of the activity was eluted in a peak following that of the HCH activity.

Precipitation with Protamine Sulfate. This agent removes nucleic acids and has been used to purify uridine diphosphate glucose pyrophosphorylase, the activity remaining in the supernatant fraction from extracts of 5-day-old etiolated shoots of Sorghum (6). The HCH activity of etiolated first internodes did not precipitate with proteinate sulfate (2 mg/ml), but only 50% of the original activity of the homogenate was recovered in the supernatant fraction. HCH activities of etiolated and green leaves, however, were almost completely precipitated by the same concentration of proteinate sulfate (Table VI), and recovery of activity in this pellet was 100% or better. So far, it has not been possible to solubilize the precipitate to permit column chromatography.

CAO activities of etiolated and green leaves were distributed quite differently by the same proteinate sulfate precipitation. Only small amounts were precipitated, and recovery was 100% or greater.

Differential Grinding to Obtain Mesophyll and Bundle Sheath Cells. Green tissues were ground briefly in an Omni-mixer in 0.5 mM sucrose, 3 mM MgCl2, 50 mM phosphate buffer at pH 7.5, to obtain an extract of mesophyll tissue. The residue from this brief cutting was well washed with the original medium and then was reground with sand in a mortar to break up the bundle sheath cells. Approximately equal activities on a gram fresh weight basis were found in both fractions, indicating that the hydroxylase activity was not restricted to cells containing chloroplasts with grana (27).

Table VI. Effect of Proteinate Sulfate Precipitation of Extracts of Green and Etiolated Shoots

<table>
<thead>
<tr>
<th></th>
<th>Green</th>
<th>HCH</th>
<th>CAO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μmoles CA/hr g fres wt</td>
<td>slope g/fresh wt</td>
</tr>
<tr>
<td>Green leaves</td>
<td></td>
<td>0.53</td>
<td>227</td>
</tr>
<tr>
<td>0-500 g Ammonium sulfate fraction</td>
<td>0.6</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Protamine sulfate-insoluble fraction</td>
<td>0</td>
<td>284</td>
<td></td>
</tr>
<tr>
<td>Protamine sulfate-soluble fraction</td>
<td>0.45</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>Etiolated leaves</td>
<td></td>
<td>0.51</td>
<td>45</td>
</tr>
<tr>
<td>0-500 g Ammonium sulfate fraction</td>
<td>0</td>
<td>247</td>
<td></td>
</tr>
</tbody>
</table>

Varied Extraction Techniques. In an effort to determine whether the grinding procedure and medium were responsible for the isolation of activity of green tissues in the 200-g ammonium sulfate fraction, different methods were used such as grinding with or without various inhibitors of polyphenolase activity (2) and rapid chromatography of crude extracts through Sephadex G-50. None of these altered the distribution pattern.

Solubilization of the Activity in the 0 to 200 g/l Ammonium Sulfate Fraction. Attempts were made to solubilize the activity in the 200 g/l ammonium sulfate fraction by conversion to a form precipitable above 200 g. Dialysis against distilled H2O, dilute buffers, or 1 mM EDTA, treatment with 0.5 mM KCl, sonication, digestion with lipases or proteinases have
not given satisfactory evidence of any conversion. But inactivation accompanies many of these treatments, complicating the interpretation. However, preliminary experiments with extraction by butanol (24), indicate a partial conversion to the 200- to 500- fraction, but only 40% of the initial activity was recovered and most of this activity was dependent upon freezing and thawing in the presence of substrate. Experiments in progress are designed to clarify this activation phenomenon.

**Determination of the Level of Caffeic Acid Esters in Etiolated and Green Second Leaves.** Previous studies indicated that greenhouse-grown Sorghum shoots contained little caffeic acid ester until after 4 to 5 days of growth, when the amount per g fresh weight increased rapidly for about 10 to 12 days (19). It was concluded that the accumulation of large amounts of caffeic acid ester in addition to those of hydroxycinnamic, ferulic, and sinapic acids was peculiar to green leaves. This analysis has been repeated with etiolated and green second leaves grown at 30 C, the same conditions used in this study for HCH activity. While caffeic acid could not be detected after alkaline hydrolysis in etiolated but expanded leaves (or less than 0.05 μmoles/g fresh weight), about 1.2 μmoles/g fresh weight were found in green leaves.

**DISCUSSION**

The sequence of enzymes producing C₆-C₃ phenolic acids from phenylalanine and tyrosine might be expected to exist as a multienzyme complex (22), possibly similar to the arom complex in Neurospora catalyzing the shikimic acid pathway (8). The large molecular weight forms of HCH reported here might be part of such a multienzyme complex, or series of complexes forming the various hydroxylated cinnamic acids, but the problem of artifacts produced during isolation altering the intracellular state or localization of enzymes remains partly unresolved (1). Mixing experiments indicate, however, that the conversion to a larger molecular weight form upon greening is not an artifact produced during grinding.

The following speculative interpretation of the sorghum data is our present working hypothesis. The HCH activity is part of a complex associated with membranes, possibly vesicles derived from the endoplasmic reticulum. Activation of the complex is dependent on attachment to binding sites on membranes associated with vacuoles, mitochondria, or plastids. The pH 8 effect demonstrated here could be due to alterations of these binding sites, but artifacts from adsorbed phenolics not removed by grinding with Dowex I have not been ruled out. Triton X-100 treatment "solubilizes" this association with a particle sedimentable at 37,000g, as has been shown in other tissues (7, 17, 24). The preliminary experiments with butanol extraction also support a membrane hypothesis (24). Association-dissociation phenomena of enzyme complexes with animal membranes are well documented (11, 16).

Extracts of etiolated and green leaves may contain a heterogeneous array of particle sizes or membrane fragments, ranging from a microsomal form to dissociating units of polymeric proteins. Since green leaves differ from etiolated ones both in the development of chloroplasts and in the accumulation of large amounts of caffeic acid ester, it is tempting to attribute the size of the particles to an association with newly formed membranes of the chloroplast, and to argue that the hydroxylase localized here might be responsible for the accumulation of caffeic acid. Certainly, this site would be efficient due to a continued supply of cofactors, whether they be NADPH, reduced pteridines, or ascorbic acid.

Recent data using electron microscopy indicate that 3-(3, 4-dihydroxyphenyl)-L-alanine oxidase is localized on granal lamellae in tobacco (4), and several investigators implicate grana as the site for both the monophenol and diphenol functions of PPO (3, 7, 15, 25). But if HCH activity of the green leaf of sorghum is associated with chloroplasts or their fragments, this association is entirely with green lamellae. After differential grinding (27) of green leaves of sorghum, the activity was present in the agranal bundle sheath fraction as well as the grana-containing mesophyll cells of this C₃ plant. Green mem- brane fragments not sedimented at 37,000g in dilute pH 6 buffer extracts can be removed completely at 100,000g, leaving about 50% of the total activity in the nongreen supernatant fraction.

The small molecular weight form of the hydroxylase found in first internodes (fraction IV from Agarose columns) could represent the basic monomeric form of the hydroxylase, although significant amounts can be found associated with a particulate fraction at pH 6 (19, 20). Internodes and etiolated leaves are similar in accumulating esters of ferulic and sinapic acid but only small amounts of caffeic acid: one of the cellular components they have in common with each other and with green leaves are vesicles derived from the endoplasmic reticulum.

In these HCH studies, only the nonester form of substrate has been used, although esters as well as free acids are considered intermediates in chlorogenic acid biosynthesis. If multi-enzyme complexes are involved, the simplest hypothesis would be to consider that only the acid or salt forms are involved and remain sequestered on the complex until the final product is esterified prior to accumulation in vacuoles or other organelles, or are transferred directly to other sites for conversion to lignin or to flavonoids via CoA-dependent steps (22, 23).

If the hypothesis of multienzyme complex(es) is correct, other enzymatic activities of the C₆-C₃ biosynthetic sequence should be present (22, 23). Evidence for the incorporation of C into ferulic acid from labeled phenylalanine and tyrosine in cell-free systems of first internode tissues has already been reported in sorghum, along with the particulate nature of cinnamic acid hydroxylase (19, 20, 22). In green leaves, the first three enzymes of the C₆-C₃ sequence (ammonia lyase, cinnamic and 4-hydroxycinnamic acid hydrolases) are associated partly or completely with very high molecular weight fractions precipitated between 50 to 200 g ammonium sulfate/1 (this paper and unpublished data). Further purification and kinetic studies of the enzymatic activities of this high molecular weight fraction are necessary to determine whether these activities are valid components of one or more multienzyme complexes.

**LITERATURE CITED**


