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# CHANGES IN PHENOLIC COMPOUNDS AND RELATED ENZYMES IN YOUNG PLANTS OF SORGHUM

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**Abstract**—The major flavonoid in vegetative green shoots of *Sorghum vulgare* var. Wheatland milo. has been identified as luteolin-7-glucoside. Minor amounts of apigenin-7-glucoside and possible diglycosides or C-glycosyl derivatives of the above flavones, as well as occasional production of the cyanidin glycoside present in seedlings, were detected. Luteolin-7-glucoside and the esters of phenolic acids, especially of caffeic acid, have similar developmental patterns, increasing to a maximum at about 10 days after germination and subsequently at a constant or slightly decreasing rate up to 30 days when based on fresh weight. Polyphenolase activity can be detected spectrophotometrically in only small amounts in etiolated seedlings; its activity begins to increase rapidly in green shoots 4–5 days after germination at a rate similar to that of the accumulation of the ester of caffeic acid. Chlorogenic acid was the most active substrate for the enzyme; others in order of decreasing activity were catechol, *p*-cresol, DOPA, and *p*-hydroxycinnamic acid. The presence of an inhibitor or latent enzyme in etiolated seedlings has not been ruled out, but the possibility of a second enzyme capable of hydroxylating *p*-hydroxycinnamic acid is discussed. Since a microsomal fraction contains phenylalanine and tyrosine ammonia lyase and cinnamic acid hydroxylase activities, this second hydroxylating enzyme of monophenols might be a part of an enzyme complex converting the amino acids to the $C_6$-$C_3$ phenolic acids.

## INTRODUCTION

A VARIETY of phenolic compounds have been identified in seedlings of *Sorghum vulgare*.[1–4] The present paper identifies the major flavonoids of green shoots as derivatives of luteolin and apigenin. These flavones accumulate under different conditions and have varied developmental patterns within sorghum plants.[4] They represent, therefore, an interesting group in terms of biochemical differentiation and regulation of the flow of phenolic compounds during the development of a plant.

Although weak cell-free activities of a few steps involving $C_{15}$ intermediates in flavonoid biosynthesis have been demonstrated with $^{14}C$ tracers,[5] the initial $C_{15}$ condensation step from $C_9$ and 3 $C_2$ precursors has not yet been demonstrated in cell-free extracts. In contrast, most of the individual steps from phenylalanine or tyrosine to the basic $C_6$-$C_3$ acids have now been demonstrated in cell-free extracts of higher plants; the ammonia lyases, the first two hydroxylating steps, transmethylation, and the formation of the respective esters via UDPG.[3, 6–10] The only missing step concerns the third hydroxylation leading to sinapic acid derivatives.

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TABLE 1. CHARACTERISTICS OF FLAVONOIDS OF THE SHOOT OF SORGHUM (MS 399)

| Spectral characteristics (13), max. in nm (italic figures = shoulder) | X−1 | | X−2 | | X−3 | X−4 |
|---|---|---|---|---|---|---|
| | Glycoside | Aglycone | Glycoside | Aglycone | Glycoside | Glycoside |
| 70% methanol | 255, *265*, 352 | 255, *265*, 346 | 268, 336 | 268, 339 | 272, 334 | 265, 271, 350 |
| +NaOH | 271, 400 | 270, *325*, 405 | 273, 294, *350*, 380 | 278, 319, 388 | 283, 334, 401 | 278, 416 |
| +NaOAc | 260, 406 | 271, 400 | 267, *365*, 390 | — | 282, *298*, 382 | 276, 282, 330, 396 |
| +$AlCl_3$ | 273, *290*, *340*, 414 | — | 276, 298, 340, 378 | — | 280, 303, 340, 380 | 277, *295*, *360*, 398 |
| +NaOAc+$H_3BO_3$ | 258, 371 | 260, 365 | 267, 340 | — | *285*, *304*, *330*, 341 | 240, *264*, 375 |
| $R_f$* BAW | 0·39 | 0·78 | 0·47 | 0·9 | 0·24 | 0·12 |
| 10% HOAc | 0·07 | — | 0·19 | — | 0·32 | 0·22 |
| Tentative identification of glycoside | Luteolin-7-glucoside† | | Apigenin-7-glucoside† | | Apigenin diglycoside‡ or *C*-glycoside | Luteolin diglycoside‡ or *C*-glycoside |
| Approximate amounts in 20-day-old shoots ($\mu$moles per shoot)§ | 1·1 | | 0·07 | | 0·1 | 0·07 |

* All dark under long u.v. and bright yellow fluorescence when fumed with $NH_3$, visible as yellow spots with $NH_3$.
† Co-chromatographed as one spot with known standards in several solvents (L-7-G from Dr. B. Brehm, A-7-G from Dr. T. A. Geissman). See Harborne.[13]
‡ Acid-resistant or no change in $R_f$ upon hydrolysis for 1 hr.
§ $\epsilon_{350} = 1{\cdot}5 \times 10^4$ for luteolin derivatives, $\epsilon_{335} = 2{\cdot}1 \times 10^4$ for apigenin derivatives.

Fresh shoots were ground in 70% methanol (v/v) followed, when necessary, by light petroleum extraction to remove chloroplast pigments. Samples for spectrophotometry were isolated as spots after two-dimensional chromatography in butanol–acetic acid–water (BAW) (6:1:2 v/v) and 10% acetic acid (HAc) (v/v). Paper blanks for spectrophotometry were generally made using the latter solvent only. Detection of compounds was based on visual characteristics in white or u.v. light, with and without fuming with $NH_3$. Co-chromatography with known standards was done with the above solvents plus a mixture of HCl: acetic acid: water (3:30:10, v/v). Acid hydrolysis was done after elution in 50% ethanol in 1 N HCl in a hot water bath for 1 hr. The original volume was maintained by the addition of ethanol. Aglycones were subsequently extracted into ethyl ether for chromatography and spectrophotometry. For analysis of spectral shifts with added reagents, the alkali was added as 0·01 ml 10 N NaOH, other agents as a few crystals directly into the cuvettes.

In preliminary studies in sorghum to determine whether the above $C_6$-$C_3$ biosynthetic sequence was present as a multienzyme complex associated with membranes of a microsomal fraction, it was observed that no classical polyphenolase activity could be demonstrated manometrically in crude extracts or ammonium sulfate precipitated fractions from etiolated seedlings, but comparable preparations from 4–5-day-old green shoots did possess an active enzyme. Polyphenolase has been postulated as the hydroxylating agent of *p*-hydroxycinnamic acid, although the activity with this substrate is weak relative to other monophenols.[9] This problem has been investigated in sorghum in relation to the developmental changes in the pattern of accumulation of phenolic compounds such as caffeic acid.

## RESULTS

### *Identification of New Flavonoids in Green Shoots*

Table 1 lists basic chromatographic and spectrophotometric characteristics of two glucosides identified as luteolin-7-glucoside, the major flavone of green shoots, and apigenin-7-glucoside, a minor component. Two other minor flavonoids are tentatively identified as di-glycosides or *C*-glycosyl derivatives of the above flavones. Trace amounts of luteolin, present as the aglycone or possibly as a breakdown product, were detected.

### *Distribution of the Major Phenolic Compounds in Sorghum Shoots During Vegetative Development*

The major phenolic compounds and their distribution during vegetative growth of the shoots (up to 1–2 months old) are summarized in Table 2. Their developmental patterns

TABLE 2. MAJOR PHENOLIC COMPOUNDS IN SORGHUM SEEDLINGS AND YOUNG VEGETATIVE PLANTS

| Compound | Root | 1st Internode Seedling stages | Coleoptile | Leaf (2–4 wk old) |
|---|---|---|---|---|
| $C_6$-$C_2$ Dhurrin | ? | Major ----------→ | | Minor |
| $C_6$-$C_3$ esters | | | | |
| pHC, Fer, (Sin) | Major----------→ | | | |
| caffeic | N.D.* or minor----------→ | | | Major |
| $(C_6$-$C_3)_n$—Lignin | Major in vascular tissue ----------→ | | | |
| $C_{15}$ glycosides | | | | |
| Apigenindin and luteolinidin | Major ----------→ | | N.D. | N.D. |
| Cyanidin | Major ----------→ | | | Minor |
| Luteolin | N.D. | Tr. | N.D. | Major |

* N.D. = not detectable. Tr = Trace

differ. Lignin and all the $C_6$-$C_3$ esters but that of caffeic acid are widespread throughout the plant at all stages. Dhurrin is synthesized only during the rapid growth of the seedling.[1,2] The 3-deoxyanthocyanins (apigeninidin and luteolinidin) are limited to non-growing portions of first internodes and roots of light- or dark-grown seedlings. The cyanidin glycoside is a

major constituent of non-growing parts of bright light-treated roots, first internodes and coleoptiles,[4, 11, 12] but accumulates only under limited conditions in mature parts of green shoots. The flavone, luteolin-7-glucoside, and the ester of caffeic acid are characteristic of growing and mature green shoots.

As might be expected from anatomical considerations, the first internode has a pattern of phenolic compounds mainly similar to that of the root, but it is shoot-like in that it contains large amounts of dhurrin. Dhurrin has been reported to be absent from roots of seedlings grown in aerated solution culture.[13] An unknown but dhurrin-like compound, however, is present in the air-grown young roots used in this study. It has similar $R_f$ characteristics, and the spectrum of its unionized and ionized form are similar to that of dhurrin.[14] However, the

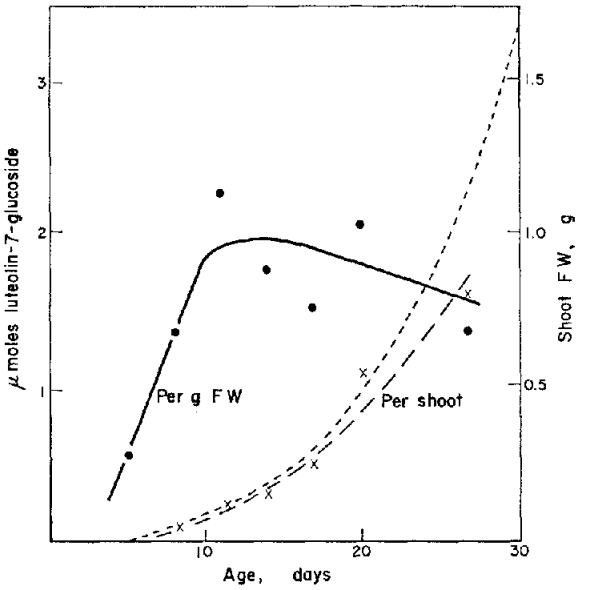


FIG. 1. CONCENTRATION OF LUTEOLIN-7-GLUCOSIDE DURING EARLY VEGETATIVE GROWTH OF SORGHUM (MS399)

●——●, μmoles luteolin per g F.W.; ×----×, μmoles per shoot; -----, F.W. of shoot. Samples were ground in 70% methanol, and after two-dimensional chromatography, spots detected at about a $R_f$ of 0·4 in butanol–acetic–water (6:1:2 v/v) and 0·07 in 10% acetic acid (v/v) were eluted in 70% methanol. Amounts are based on $\epsilon_{350}$ of $1{\cdot}5 \times 10^4$. (N = 1.)

anion is stable in alkali, with only traces of the peak at 330 nm due to the production of *p*-hydroxybenzaldehyde from dhurrin.

Not all cells accumulate the above array of phenolic compounds. Experiments in which cortical cells were stripped from the stele with a wire-cutter indicate that the flavonoids and dhurrin were limited to the cortex and lignin to walls of the central stele, while the esters of phenolic acids appear in both parts. Observation of fresh hand-cut cross sections indicate that the cyanidin derivative appears first in the vacuoles of sub-epidermal cells and spreads

[11] R. J. DOWNS and H. W. SIEGELMAN, *Plant Physiol.* **38**, 25 (1963).
[12] H. A. STAFFORD, *Plant Physiol.* **43**, 318 (1968).
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[14] C. H. MAO, J. P. BLOCHER, L. ANDERSON and D. C. SMITH, *Phytochem.* **4**, 297 (1965).

inward. Apigeninidin and luteolinidin are much harder to observe, but presumably also accumulate in the vacuole along with esters of phenolic acids and dhurrin; however, it is not known if any one cortical cell can make all these types of compounds.

*Developmental Aspects of Accumulation of Luteolin Glucoside and the Esters of Caffeic and Related Acids*

Figure 1 shows the rate of accumulation of luteolin-7-glucoside during the growth of the shoot (mainly made up of leaves). It increases more rapidly than the fresh weight of the shoots

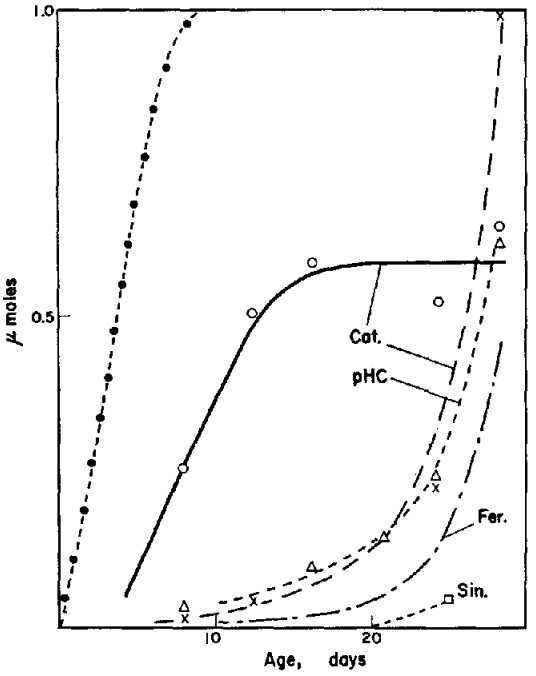


FIG. 2. CONCENTRATION OF PHENOLIC ESTERS DURING EARLY VEGETATIVE GROWTH OF SORGHUM (OKLA. STRAIN).

Caffeic acid, ○——○ μmoles per g F.W.; ×---× μmoles per shoot. *p*-Hydroxycinnamic pHC, △---△; ferulic, -·-·; sinapic, ....□; and dhurrin, ●---●, all shown only as μmoles per shoot. Samples were extracted in hot 70% methanol. After extraction with light petroleum, the methanol extract was concentrated under vacuum at 50° and hydrolyzed in 2 N NaOH for 5 hr in an evacuated desiccator. After acidification and extraction in ethyl ether, the samples were chromatographed in benzene–acetic acid–water (4:1:0·1) and Na formate–formic acid–water (2·5 g:0·25 ml:50 ml). A strip of the two-dimensional chromatogram containing ferulic and sinapic acid area was cut off and re-chromatographed in the latter to complete separation. The fluorescence was determined in alkaline Tris or NaOH.[12] (N = 1.)

for about the first 10 days, and then remains constant or decreases slightly for the rest of the first month of growth. The amounts on a per shoot basis are however still increasing after 1 month of growth.

Figure 2 shows that the accumulation of the $C_6$-$C_3$ esters of the hydroxycinnamic acids follows a pattern similar to that of the flavone glucoside. Identification of the sinapic acid ester and its separation from that of ferulic acid is difficult, and the values given may not be exact. Unlike first internodes,[4] the ester of caffeic acid accumulates in older plants to a greater

extent than the other esters; the amount formed is variable. Values obtained in separate analyses from 1–3-month-old greenhouse-grown plants vary from these shown to ones as high as approximately 1·8 μmoles per g fresh weight. The shoots are still accumulating these esters at 28 days of growth at an approximately linear rate per shoot. Factors controlling the accumulation of these compounds need further study.

Comparison of the accumulation of these esters with that of the $C_6$-$C_2$ cyanogenic glucoside, dhurrin, indicates a strikingly different pattern of accumulation during development. The amount of dhurrin per g fresh weight has been reported by Akazawa and Conn[1] as being not detectable in dormant seeds and rising to about 30 μmoles per g fresh weight at about 5 days of growth in the light in an aerated solution culture. The high values are partly a reflection of the difference in fresh weight per plant in the two culture methods. But, in contrast to the above esters and flavones, the value decreased to about 8 μmoles per g fresh weight at 25 days. Similar results were obtained by Lawlor, 1966 (unpublished Reed College Senior Thesis). The approximate rate of increase on a per shoot basis is shown to the left of the graph in Fig. 2; the peak was at about 1·2 μmoles per shoot at 5–8 days of growth in solution culture, remaining constant or dropping sightly during the rest of the first month of growth. Dhurrin, therefore, is rapidly synthesized or accumulated only in the very early stages of growth of the shoot. The fate of the dhurrin formed has never been established.

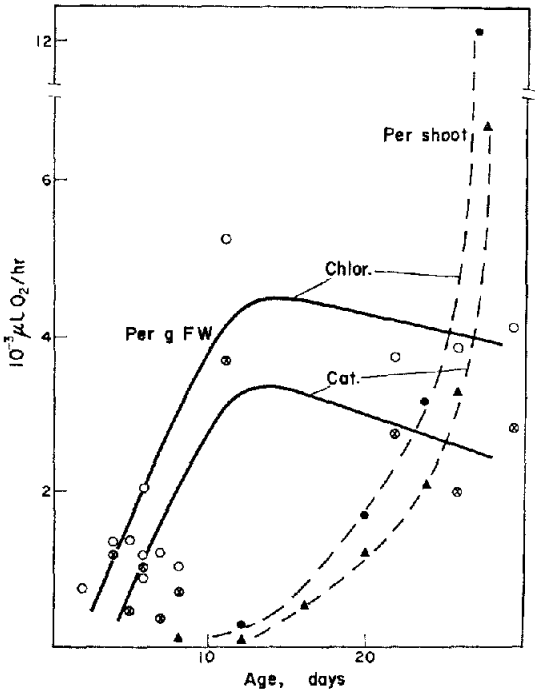


FIG. 3. POLYPHENOLASE ACTIVITY (MANOMETRIC ASSAY) DURING EARLY STAGES OF VEGETATIVE GROWTH OF SORGHUM,

Chlorogenic acid, ○——○ per g F.W.; ●----● per shoot: catechol ⊗——⊗ per g F.W. and ▲——▲ per shoot. Each Warburg vessel contained in a 3 ml volume; 2 ml enzyme, 150 μmoles phosphate buffer, pH 6·8, 30 μmoles of chlorogenic acid or catechol. (N = 2.) Boiled controls were subtracted. The rate was linear for 30 min, subsequently falling off rapidly. A crude enzyme extract was prepared by grinding approximately 2·5 g F.W. shoots in 10 ml 0·05 M phosphate buffer at pH 6·8. After straining through cheesecloth, the filtrate was used directly.

*Polyphenolase Activity*

No polyphenolase activity could be detected manometrically in dark-grown or green young shoots or in 1-month-old soil-grown roots, after extraction in phosphate or Tris buffers with or without subsequent dialysis or precipitation with ammonium sulfate. Active preparations, however, were obtained in green shoots after 4–5 days of growth (Fig. 3). The increase in activity with growth of the green shoot closely paralleled that of the accumulation of the ester of caffeic acid. There was a decreasing order of activity with the following substrates; chlorogenic acid, catechol, DOPA, *p*-cresol, the latter being very weak.

This lack of activity in etiolated seedlings is an anomaly since this enzyme has been postulated to be the hydroxylating agent of monophenols.[9] Since diphenols are actively synthesized in the same tissue from endogenous precursors or when incubated with phenylalanine or tyrosine, the lack of activity means either that the enzyme is inactivated in cell-free extracts, or that there is a second enzyme that is the major physiological agent of this hydroxylation.

Some information has been obtained concerning this problem. There is evidence that an active leaf preparation is inhibited by inactive internode extracts when tested manometrically (unpublished data of Nancy Adams, Reed College Senior Thesis, 1968). This inhibiting factor is heat unstable, and therefore could be due to an enzymatic activity or to an unstable substrate.

On the other hand, using the more rapid and sensitive indirect spectrophotometric method,[9] a weak activity with chlorogenic acid, approximately 1/1000 that of a comparable shoot preparation, can be demonstrated if dialyzed buffer extracts of first internodes are used (Table 3). Evidence of an inhibitor in dialyzed extracts by mixing internode extracts with more active shoot preparations cannot be shown with this spectrophotometric assay even after preincubation of the enzyme mixture before substrates were added. A variety of treatments, ammonium sulfate, protamine sulfate, urea activation, as well as a dialyzed extract of an acetone powder, have not given any activities above 500 units for chlorogenic acid oxidation. Comparable preparations from different batches of internodes are quite variable, ranging from about 50 to 500 units. Infiltration of internodes with 1 $\mu$M or 1 mM chlorogenic acid, and subsequent incubation for 16 hr in bright light before extraction, did not give any significant increase in activity. Extraction in the presence of 1 mM chlorogenic acid inhibited the activity. Determination of the intracellular localization of this weak activity has not been feasible. No detectable activity was obtained with phenolic substrates other than chlorogenic acid.

When crude preparations of shoots were assayed spectrophotometrically, there was a decreasing order of activity with the following substrates: chlorogenic acid, catechol, *p*-cresol, DOPA, *p*-hydroxycinnamic acid (relative activities of 100:40:10:2:not detectable). In a fraction purified on a DEAE column, the relative activities were 100:100:10:6:1. Rates for monophenols were obtained after a lag period of 20–30 min. Activity with tyrosine was not detectable.

*Related Enzyme Activities*

*Ammonia lyases.* Data are shown in Table 3 to indicate that there are also difficulties in obtaining active cell-free preparations that deaminate phenylalanine and tyrosine to their respective cinnamic and *p*-hydroxycinnamic acids, but that the problem in first internode and green-shoot tissues is just the reverse of that for the polyphenolase enzyme.

While acetone powder extracts (plus 2-mercaptoethanol in the case of shoots) are of comparable activity for the first internode and green-shoot preparations, buffer extracts of fresh

internodes ground with Polyclar are much more active than comparable extracts of green tissues. GSH appears to be a slight protecting agent in the case of the first internodes, while mercaptoethanol was essential and the best protecting agent found so far with shoot preparations. Approximately one-third of the total activity was associated with a high-speed frac-

TABLE 3. ACTIVITIES OF POLYPHENOLASE AND AMMONIA LYASES IN FIRST INTERNODES AND 2–4-WEEK-OLD SHOOTS

| | First internodes (units per g F.W.) | | Shoots (units per g F.W.) | |
|---|---|---|---|---|
| Chlorogenic-acid oxidase | 300–500 | | 30,000 | |
| Ammonia lyases | Phenylalanine | Tyrosine | Phenylalanine | Tyrosine |
| | μmoles cinnamic or pHC per g F.W. | | | |
| Acetone powder | | | | |
| Tris-EDTA-GSH | 384 | Tr. | — | — |
| Tris-EDTA-ME | — | — | 404 | 43 |
| Buffer extract | | | | |
| Tris-EDTA-GSH | | | | |
| 100,000 × *g** | 122 | 11 | — | — |
| Supernatant | 184 | 21 | — | — |
| Tris-EDTA-ME | | | | |
| 100,000 × *g* | — | — | 20 | Tr. |
| Supernatant | — | — | 70 | Tr. |

* Similar activities if 0·5 M sucrose present, and about 40 per cent of this activity was associated with a particulate preparation centrifuged 30 min at 18,000 × *g*, with the remaining 60 per cent with the subsequent fraction obtained at 100,000 × *g* for 2 hr.

Polyphenolase activity was determined at room temperature by the indirect spectrophotometric method by following the oxidation of ascorbic acid at 265 nm for the first 3–5 min.[9] Total units = $10^3$ ΔO.D./min. Each cuvette contained 1 mM $Na_2EDTA$, 180 μM chlorogenic acid, 28 μM ascorbic acid, 30 μM phosphate buffer at pH 6·8. The reference cuvette contained all but chlorogenic acid. The reaction was started by adding the enzyme to both cuvettes. The enzyme preparations were either dialyzed phosphate buffer extracts (pH 6·8) or fractions precipitated with ammonium sulfate (200–500 g A.S. per l.) and resuspended in phosphate buffer.

Activity of ammonia lyases was analyzed spectrophotometrically after chromatography of a mixture incubated for 2 hr with shaking at 30° in 50 mM Tris buffer, pH 8·4, 5 mM tyrosine or phenylalanine, 5 mM $Na_2EDTA$, 3 mM GSH or 1 mM 2-mercaptoethanol (M.E.). After acidification, the products were extracted with ethyl acetate and chromatographed in benzene–acetic acid–water (4:1:0·1) and Na formate–formic acid–water (2·5 g:0·25 ml:50 ml). Spots identified by fluorescence under u.v. were eluted in 1·5 ml methanol or methanol + 0·05 ml 1 N NaOH and analyzed spectrophotometrically at 270 and 330 nm for cinnamic and *p*-hydroxycinnamic acid respectively. Calculations were based on standards taken through the same procedure. All enzyme extracts were made in pH 8·4 Tris buffer plus 0·1 by weight of Polyclar AT and EDTA and GSH or M.E. as indicated above. After straining through cheesecloth, they were centrifuged at 500 × *g* for 1 min.

tion (500–100,000 × *g*) that included mitochondria and possible other cytosomes as well as microsomes. Even in the more active internode preparations, there was evidence of variability in the supernatant fraction, and linearity was obtained only over very narrow enzyme concentration ranges. The reaction was linear, however, up to 3 hr. High blanks in preparations incubated with enzyme alone, even in the microsomal fraction, prevented the use of

a direct spectrophotometric assay of the ethyl acetate extract, and necessitated the use of chromatographic procedures to isolate the products; the presence of the substrate altered the amounts of these interfering compounds. The activity with tyrosine was always roughly one-tenth that with phenylalanine. This was not expected from data using $^{14}C$ tracers in which tyrosine was sometimes as good a precursor of flavonoids as phenylalanine (unpublished data). This might mean either that there was a specific inactivation of the tyrosine ammonia lyase, or a regulatory phenomena in intact cells controlling the rate of the two enzymes.

*Cinnamic acid hydroxylase.* Preliminary experiments with and without radioactive tracers have demonstrated that a 100,000 × *g* fraction obtained from tissues ground in pH 7·5 50 mM phosphate buffer contains all the cinnamic hydroxylase activity of the original extract. The components and characteristics of the system were similar to those described by Russell and Conn in peas.[10]

## DISCUSSION

Factors controlling the biochemical differentiation of phenolic compounds in sorghum plants have been only partially explored. High intensity blue light is obligatory and red light is subsequently regulatory in cyanidin biosynthesis.[4,11] An oxygen requirement varies from a high one for dhurrin[2] to slightly less in a decreasing series for cyanidin, the two 3-deoxyanthocyanins and phenolic esters, and least for lignin formation.[4] An oxygen gradient due to the absence of stomates and a closer packing of cells in the solid cylinder of tissue of roots and the first internode might account for some of the above restriction.

The problem of the low activity of a classical polyphenolase in first internodes in contrast to that of green shoots and its possible relationship to the increase in accumulation of the ester of caffeic acid, is still under investigation. While it is possible that inactivation or presence of a latent form is the cause of the weak activity, it is also conceivable that two hydroxylating enzymes for monophenols may be involved, one associated with a microsomal fraction, the other with chloroplasts. The latter would also have the diphenol function of a classical polyphenolase.

The enzymes concerned with the series of reactions leading to the closely related sequences of $C_6$-$C_3$ and $C_{15}$ compounds from phenylalanine and tyrosine may exist as a multienzyme complex. Since cinnamic hydroxylase is limited to a high-speed particulate fraction (Ref. 10, and this paper), some of the other activities should be found there, unless they are easily washed off. The two ammonia lyases are partly associated with this fraction in extracts from sorghum. The complex in this 100,000 × *g* fraction could actually be in cytosomes such as lysosomes or peroxisomes which ultimately release their substrates into the central vacuole.

Some of the activities might also be part of an organelle such as a plastid. Green chloroplasts are never found in the root, first internode or coleoptile of sorghum even in light, but carotenoids and leucoplasts or starch grains are present.[4,12] The green leaves do contain chloroplasts and, as expected from data from other tissues,[15] much of the polyphenolase activity (chlorogenic acid and catechol) in sorghum shoots prepared in sucrose media was associated with intact chloroplasts (unpublished data). Chloroplasts have been postulated as the site of hydroxylation of *p*-hydroxycinnamic to caffeic acid and subsequent conversion to esculetin.[15,16] It will be of interest to determine whether the formation of the caffeic ester

[15] M. SATO, *Phytochem.* **6**, 1363 (1967).
[16] M. SATO, *Phytochem.* **5**, 385 (1966).

and possibly luteolin-7-glucoside may be associated with organized chloroplasts in sorghum, accounting for the accumulation of the large amounts of these compounds in green shoots in contrast to etiolated seedlings. Furthermore, it is possible that the origin of the ferulic ester (and subsequently sinapic ester) could be via a direct methoxylation, rather than a stepwise hydroxylation followed by methylation. But the presence in chloroplasts of a second hydroxylating activity (phenolase complex), unaccompanied by a methylating system, would give rise to the same results, i.e. the accumulation of caffeic ester that is independent of that of other cinnamic acid esters. Leaves, therefore, might have two major hydroxylating systems for monophenols, one of which is in chloroplasts. Internodes and roots may possess only small amounts of the one hydroxylating enzyme (phenolase complex) associated with carotenoid-containing proplastids or leucoplasts, but large amounts of the second hydroxylation system associated with a methylating system in the microsomal fraction. Efforts are now being made to determine whether there is a separate hydroxylating system in the microsomal fraction, and whether it may require a pteridine co-factor as in the tyrosine hydroxylase in animal tissue.[17]

The study of the biosynthesis of phenolic compounds in sorghum may help to answer the question of whether the phenolic acids or their esters are ever true intermediates of the compound next in the series, and whether there may be only one or a series of complexes leading to each product that is accumulated. The possibility of one or more multi-enzyme complexes in a microsomal or cytosomal fraction converting phenylalanine and tyrosine sequentially to the hydroxylated and methylated phenolic acids or their esters is being actively studied at present.

## GENERAL METHODS

Plants and culture conditions: Seeds of *Sorghum vulgare* variety Wheatland milo, were obtained from the Great Plains Field Station, U.S.D.A., at Woodward, Oklahoma (Oklahoma strain), and from Texas Agricultural Experiment Station, College Station, Texas (MS 399). Plants were grown in a growth chamber at 30,000 to 40,000 lux of white fluorescent light at 23 to 25° or in a greenhouse in soil or in vermiculite with an inorganic nutrient supplement (Hygro) after about 2 weeks of growth. Seedlings for first internodes were grown on filter paper for 3–4 days in the dark as described in previous papers.[4, 12] Specific methods are described under figures or tables.

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